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Yersinia enterocolitica in Aquatic Environments

Development and Assessment of a Membrane Filtration Procedure

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YERSINIA ENTEROCOLITICA IN AQUATIC ENVIRONMENTS

Development and Assessment of a Membrane Filtration Procedure

K.P. Lautenschlager

A.T. Kafal

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FOREWORD

The following report has been prepared mainly to provide a detailed description of a laboratory and field assessment of a membrane filtration procedure for the enumeration of Yersinia enterocolitica-like bacteria in aquatic environments.

The report is intended to assist laboratory staff in developing a proficiency in isolating yersiniae from water and sewage, and from animal sources. It should also serve as a useful guide in the selection of techniques required for assessing the performance characteristics of other new procedures, as well as for procedures that are currently used routinely for bacteriological examinations of water.

K.P. Lautenschlager
Supervisor
Quality Assessment Section

ABSTRACT

During a study of pathogenic bacteria in recreational lakes of the Muskoka-Haliburton region of Ontario several published methods for the isolation of Yersinia enterocolitica (Y.e.) from water were assessed during the period 1978 to 1980. Concurrently a two-step membrane filtration procedure was developed and applied to routine determinations of Y.e. in more than 600 surface water and 80 wastewater samples from February 1979 to January 1980. This procedure consisted of primary incubation of membrane filters on Rimler-Shotts broth (RSB) for 22 h at 35°C with subsequent transfer of filters to Christensen's urea agar (CUA) for secondary incubation for 24-48 h at 35°C. By utilizing a restricted target colony description the specificity of the RSB-CUA system was estimated to be 86% after confirmation of 545 of 633 target colonies as Y.e. using the API-20E biochemical test system. Presumptive and confirmed selectivity indices were calculated for cumulative results for observed target and background colony counts on 834 membrane filters used to analyse 545 surface water samples. Presumptive selectivity of the RSB-CUA system ranged from 0.1844 during periods of low water temperature (1.0°C) to 0.0000 in August and September when water temperatures usually exceeded 15°C and levels of background micro-organisms were greatest. On individual membrane filters, however, target colonies frequently were observed to comprise up to 50% of the total colonies recovered during winter sampling periods. False presumptive target colonies comprised 14% of cumulative observed target colonies on filters and these were mostly encountered in samples analysed during summer months. This false target colony population was comprised

mostly of species of Enterobacter, Klebsiella, Aeromonas, Hafnia, Pseudomonas, Providencia and Serratia. Of the confirmed Y.e. isolates obtained with the RSB-CUA system and subjected to serological characterization 41% (73) were serotypable and these constituted 18 distinct serological groups. Efforts to improve the selectivity of the RSB-CUA system for Y.e. using increased concentrations of antibiotic or deoxycholate or by altering incubation regimes were not successful. The system, however, is one of the few available procedures for detection and quantitation of Y.e. and Y.e.-like bacteria in lake, river and well waters and in wastewaters of sewage treatment systems.

One cold-enrichment procedure utilizing urea deoxycholate broth and MacConkey Tween agar was assessed and was found useful in the detection of Y.e. from water and from the intestinal contents of Castor canadensis. The same procedure also provided the means for the isolation of the first known mammalian enteric strain of Y. ruckeri from muskrat (Ondatra zibethica).

Recommendations for the application and improvement of quantitative procedures for the analyses of water samples for Y.e. are presented.

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Chapter I

Introduction

In recent years Yersinia enterocolitica (Y.e.) has gained prominence as an increasingly important pathogen of humans (Toma and Lafleur 1974; W.H.O., 1976; Bottone 1977; Kohl 1978, 1980). Its association with outbreaks of human illness, mainly gastroenteritis, has stimulated much research into the ecology and physiology of this bacterium but the pathogenisis of Y.e. in human illnesses remains poorly understood (Mair 1973, Bottone 1977). Despite numerous outbreaks of gastroenteritis due to Y.e. in which foods were considered to be the most likely vector of the organism only one epidemiological investigation (Black et al 1978) has conclusively demonstrated food as the source of this etiological agent. Y.e. are widely distributed in aquatic environments (Kapperud and Jonsson 1978) and waterborne strains have been implicated but not confirmed as the etiological agent responsible for several hundred cases of gastroenteritis at a ski resort in Montana (Eden et al 1977). Their involvement in human disease has been more strongly implicated by reports of single cases of gastroenteritis (Lassen 1972) and in near fatal septicemia (Keet 1974) and by the demonstration of the pathogenicity of some water isolates of Y.e. for laboratory animals (Carter et al 1973; Raevuori et al 1979; Schiemann and Devenich 1980). Despite this apparent potential for involvement in human disease epidemiological studies in most cases have failed to identify the specific sources of Y.e. involved in outbreaks of disease. That this has been so may be mostly attributable to the current lack of suitable methods for detecting Y.e. in environmental samples and in foods. At the time the present study was conducted no reports of quantitative procedures

for the determination of Y.e. in aquatic environments or in other potential sources of Y.e. had appeared in the literature. For these reasons a study of Y.e. in recreational waters of the Muskoka - Haliburton areas of central Ontario was initiated in 1978 by the Microbiology Component of the Lakeshore Capacity Study.

During that study several procedures for the detection of Y.e. in water and in wildlife feces were assessed and through a series of serendipitous discoveries a membrane filtration system for use in the quantitation of Y.e. in water was developed. Some of the findings of the application of this membrane filtration procedure to the analysis of surface, well,and waste waters have previously been reported (Lautenschlager 1980). In the present report the results of experiments designed to assess various procedures for detecting or quantifying Y.e. in environmental samples are presented.

Chapter II

Literature Review

A preliminary review of methods concerning published methods which were thought to be of potential practical value in the study of Y.e. in recreational waters was prepared at the start of the present study (Lautenschlager 1978). Since that time several new procedures and enteric media have been reported in the literature as new tools for the study of Y.e. in water, in foods, and in clinical specimens. The following literature review was prepared to provide a summary of some of these new methodological developments and to discuss their possible applications to the study of Y.e. in aquatic environments.

Most of the recently published procedures for detecting Y.e. in fecal or environmental samples have been developed to recover the strains of Y.e. which are most commonly associated with human infections. In Canada, Europe and Scandinavia these strains are mostly serotypes 0:3; 0:9; 0:8; 0:6,30; 0:4,32 and 0:5,27 although numerous other serotypes of Y.e. have been reported from clinical specimens as well (Toma et al 1979). The biochemical characteristics of these commonly encountered clinical strains have been studied extensively (Nilehn 1969b,c; Winblad 1972; Brenner et al 1976; Bottone 1977; Pedersen et al 1979) in an effort to distinguish some biochemical characteristics which may be useful as epidemiological markers indicative of strains which are of potential significance to human health. Studies of this kind have led to the suggestion of correlations between biochemical or serological characteristics of certain groups or strains of

Y.e. with the ability of Y.e. to produce heat stable enterotoxin (Pai et al 1978), to invade HeLa cells (Une et al 1977; Maruyama et al 1979), to the production of haemagglutinins (Maclagan and Old 1980), to the production of hemolysin (Tsubokura et al 1979) and to the production of factors involved in the virulence of Y.e. (Schiemann and Devenish 1980). Few isolates of waterborne Y.e. have been examined in these regards so these suggested correlations may apply only to specific groups of human Y.e. isolates and not to the diverse population of strains of which the species is comprised. Methods which have recently been developed to recover mostly strains of Y.e. which are "typical" with respect to meeting the criteria upon which these correlations were developed may inadvertantly fail to detect other strains of Y.e. which, although potentially pathogenic to humans (see Bottone 1977), are biochemically or serologically atypical of the strains which have historically been most often involved in cases of human yersiniosis. Owing to the numerous temperature-dependent physiological (Nilehn 1969a, 1973; Carter and Collins 1974; Maclagan and Old 1980; Zink et al 1980) and biochemical (Nilehn 1969b; Chester and Stotsky 1976; Bottone 1977) characteristics of this species and the potential of Y.e. to acquire new characteristics by intraspecific and interspecific conjugation (i.e. Bechtel and Boring 1979) or by acquisition of plasmids (Zink et al 1980) the procedures and cultural conditions used to isolate strains of Y.e. must be carefully studied to avoid the development of conclusions regarding species characteristics which may in reality apply only to specific strains or groups of strains of this species. These somewhat philosophical considerations may be of secondary importance to the development of methods for detecting Y.e. in

environmental or clinical samples but they may be of profound significance to the development of epidemiological tools for accurately determining the relationship between environmental Y.e. and human or animal health.

Clinical isolates of Y.e. have mostly been obtained through the use of enrichment culturing techniques in which feces or other clinical specimens have been incubated in suspension with a selective medium, by incubation at low temperature in a buffered water suspension (Eiss 1975; Pai et al 1979), or by direct inoculation of specimen onto a selective enteric medium (Pai et al 1979). Recently a number of enteric media have been reported to afford excellent rates of detection of Y.e. in fecal samples when used for direct isolation or following cold-enrichment of the specimen (Juhlin and Ericson 1961; Christenson and Jansen 1977; Dudley and Shotts 1979; Schiemann 1979; Soltesz et al 1980). Similar procedures have been employed for detecting Y.e. in foods (Mehlman et al 1978; Aulisio et al 1980; Lee et al 1980), soil (Barre et al 1979) and water (Prior 1976; Kapperud 1977). Although Y.e. isolation protocols utilizing a cold-enrichment procedure have been widely used, especially for fecal analyses, the low-temperature culturing technique is apparently not always required when samples of feces are derived from symptomatic patients (Pai et al 1979).

In recent studies of Y.e. in well or surface waters Y.e. were recovered from among the background colonies observed on membrane filters used to analyse water samples for total coliform bacteria. Highsmith et al (1977) found several "distinct, small dark red colonies" growing in the background on filters incubated at 35°C on M-Endo medium to be strains of Y.e.

Similarly Schiemann (1978) reported the detection of Y.e. as tiny, drop-like mostly colorless colonies on membrane filters incubated on M-Endo agar LES (BBL) but exposed to a different incubation regime than was employed by Highsmith et al (1977). The study of Highsmith et al (1977) also recommended that in the most probable number tests for coliforms, when negative presumptive tests are obtained but tubes of lactose broth are turbid, cultures in these tubes should be examined further and tested for the presence of Y.e. At the outset of the present study neither of these procedures were considered for application to the detection of Y.e. in recreational waters due to the lack of specificity of these media for Y.e., and because of our familiarity with the frequently observed profusion of tiny red or colorless background colonies recovered during membrane filtration analyses of water for coliforms (Ministry of the Environment 1980). However, several other procedures which had provided the means for isolating Y.e. from lake water for other researchers were adopted for further study.

Harvey et al (1976) and Pickett (1977) reported success in isolating Y.e. from streams and lake water of mountainous areas of California by using a combination of enrichment incubation of water samples and a variety of selective isolation media. These procedures like those already described above, however, did not provide either a method which could be used routinely nor the means to quantitate Y.e. in water samples. Never-the-less much effort was made to assess and to improve upon the methods reported by Harvey et al (1976) and Pickett (1977) while at the same time developing and applying a membrane filtration procedure to waters of the

Lakeshore Capacity Study area. The following presentation describes the procedures which were employed for isolating and/or quantifying Y.e. in surface, well and waste waters of central Ontario by the Microbiology Component. Considerable emphasis has been made with regard to a membrane filtration procedure developed by this component for determining levels of Y.e. in waters. Estimates of some of the performance characteristics of the RSB-CUA membrane filtration system also are presented.

Since preparing the manuscript for the current presentation the senior author became aware of two other research groups that have, independently, developed and applied various procedures for the isolation and/or quantitation of Y.e.-like bacteria from aquatic samples. One procedure, a membrane filtration technique, was developed by T.D. Bartley, T.J. Quan, M.T. Collins and S.M. Morrison (1982, in press, Applied and Environmental Microbiology). Several other procedures have recently been described by T.F. Wetzler and G.J. Ma at the American Water Works Association Water Quality Technology Conference in Seattle, December 6-8, 1981.

Chapter III

Materials and Methods

General Information

Procedures employed in the collection of water and sediment samples, in membrane filtration analyses of water samples, and in the maintenance, frozen preservation, and identification of bacterial isolates have been described in detail in a previous unpublished report (Ministry of the Environment, 1980, in press). All membrane filtration analyses conducted on water samples were performed using Gelman, type GN-6 (0.45 micron pore size) membrane filters (MF) with 3 mm black-gridded working surfaces. The sampling sites utilized in this study have been described or listed in another report (Lautenschlager 1980).

Screening procedures employed for bacterial isolates prior to their identification using a commercially available biochemical test system (API-20E, Analytab Products, New York) included tests for motility in motility sulfide medium (Difco) conducted at 25 and 35°C, urease reaction on slants of Christensen's area agar (Difco) at 35°C, oxidase reaction on TSA plates, growth and reaction on triple sugar iron agar (Difco) slants at 25°C and growth on MacConkey agar (Difco) at 35°C. Y.e. strains often are: non-motile at 35°C but motile at 25°C, oxidase negative, urease positive, produce acid slant and butt reactions usually without gas and no H_2S production in triple sugar iron agar, and grow on MacConkey agar at 35°C. A similar battery of screening tests has been employed in other studies following the isolation of Y.e. from water (Highsmith et al 1977; Harvey et al 1976).

Observations of the colonial morphological features of Y.e. and other bacterial colonies growing on membrane filters or on plates of agar media were made using a steroscopic microscope with an oblique fluorescent light source.

Media

MacConkey agar (Difco) was frequently employed as a medium in screening tests prior to identification of bacterial isolates using the API-20E biochemical system. MacConkey Tween 80 (MT80) was used as a recovery medium for isolation and purification of isolates from enrichment media and from the surfaces of membrane filters. MT80 was prepared using the manufacturer's recipe for MacConkey agar (Difco) with the addition of Tween 80 (Difco), just prior to the sterilization procedure, to give a final concentration of Tween 80 of 1.0% (v/v). Calcium chloride was not included in its formulation. Christensen's urea agar, CUA (Difco), used in screening procedures and as a secondary isolation medium in the membrane filtration procedure for Y.e., was prepared according to the manufacturer's recipe with filter-sterilization of the urea agar base solution carried out using Sartorius (type 11106) membrane filters (0.45 micron pore size). Modified CUA containing maltose instead of glucose as the sole carbohydrate source was later tested as an alternate isolation medium in the membrane filtration procedure for Y.e. This medium, mCUAM, was prepared in the folowing manner: Urea agar base solution, consisting of peptone (Difco), 1.0 g; maltose (Difco), 1.0 g; sodium chloride, 5.0 g; KH_2PO_4, 2.0 g; phenol red (Matheson, BDH), 0.012 g; urea (Difco), 20.0 g dissolved in 100 ml distilled water, was filter-sterilized as described above. Another solution was prepared by dissolving 15.0 g of agar (Difco) in 900 ml

distilled water by heating. This solution was sterilized by autoclaving for 15 minutes at 121°C, cooled to 50°C and then aseptically mixed with the filter-sterilized urea agar base solution. The final pH was adjusted, after mixing of the solutions, to pH 6.8 at 25°C. For use as isolation media MT8O, CUA and mCUAM were dispensed in 20-25 ml quantities into 90 mm diameter sterile disposable plastic petri dishes and stored at 4°C for up to 3 weeks or until used experimentally.

Urea deoxycholate broth, UDB, was used as a selective-enrichment medium for the attempted recovery of Y.e. from water and sediment samples and from intestinal contents of wildlife specimens. A similar formulation of this medium, UDB, was previously employed by the working group of Pickett (1977) as an enrichment medium for the recovery of Y.e. from lake water in California. In the present study UDB was prepared by dissolving 77.4 g dehydrated urea broth (Difco) together with 2.0 g of sodium deoxycholate (Difco) in 1,000 ml distilled water. This double strength solution was then filter-sterilized, and aseptically dispensed in 5 ml volumes to sterile screw-capped glass tubes, and then stored at 4°C without apparent change in the medium for up to 30 days or until required. When used for analysis, tubes of UDB were inoculated with 5.0 ml of water or other sample material to produce a final sodium deoxycholate concentration of approximately 0.1%. In some experiments, to be described below, additional quantities of sodium deoxycholate were added to the medium prior to filter-sterilization to increase the final concentration of this inhibitor in inoculated tubes of UDB.

Most membrane filtration analyses of water samples were carried out using Rimler-Shotts broth (RSB) as the primary isolation medium for recovery of Y.e. Originally used as a membrane filtration medium for the determination of Aeromonas hydrophila in water by Fliermans et al (1977) a slightly modified formulation of this broth medium was employed in the present study. Rimler-Shotts agar, RSA, originally designed for isolation of Aeromonas hydrophila from water by Shotts and Rimler (1973), was also employed experimentally in this study as a primary isolation medium in the membrane filtration analysis of water samples for Y.e.

In the present study RSB was prepared by the following procedures. Quantities of various ingredients, L-lysine HCl, 5 g; L-ornithine HCl, 6.5 g; maltose, 3.5 g; sodium thiosulfate, 6.8; L-cysteine HCl, 0.3 g; bromothymol blue, 0.03 g; ferric ammonium citrate, 0.8 g; sodium deoxycholate, 1.0 g; novobiocin, 0.005 g; yeast extract, 3.0 g; and NaCl, 5.0 g were dissolved in a sufficient amount of distilled water to make 1 L of medium, and the pH of the solution adjusted to 7.0. The solution was then heated to boiling, boiled for 1 minute then cooled to 45°C, aseptically dispensed in 100 ml volumes to sterile glass bottles, and then stored at 4°C for up to 2 weeks before being used for analyses of samples. RSA was prepared in a similar manner but 15 g rather than 13.5 g, of agar (Difco) was added to the mixture prior to boiling. The agar formulation also differed from that developed by Shotts and Rimler (1973) in that 1,000 ml of distilled water was used to dissolve the ingredients rather than just adding sufficient water to make 1 L of medium.

In some experiments, to be described below, additional quantities of sodium deoxycholate or novobiocin were added to RSB during its preparation. During membrane filtration analysis RSB was aseptically dispensed in 4 ml amounts to pads of absorbant paper (Fliermans et al 1977) housed in 50 mm diameter disposable plastic petri dishes just prior to the actual analysis. Excess RSB was decanted from these dishes before MF was placed upon the saturated paper pads. When RSA was employed in these analyses MF were placed directly upon the surface of RSA which had previously been poured into large, square, disposal plastic dishes. Usually 2 MF were placed upon medium contained in the larger dishes of RSA.

Preliminary observations made during the assessment of RSB for use in membrane filtration analyses showed that some pads of absorbant paper caused a rapid, apparent reduction in the pH of RSB as the broth contacted the paper. This resulted in a colour change in the medium from green-blue to pale green or yellow. For this reason tests were carried out to ensure that available lots of paper pads did not cause this acidic reaction and large quantities of one lot of pads which caused no such reaction were obtained and used for all subsequent analyses with RSB. Except as specified for only a few experiments, all membrane filters which were incubated on RSB were incubated at 35°C for exactly 22h and then observed for counts of various bacteria. Delays in observing colonies at that time or prolonging the period of incubation was found to result in colour reversions and changes in colonies as previously described for RSA (Shotts and Rimler 1973).

In some experiments where anaerobic conditions were required during the incubation of various tubes of inoculated media or of membrane filters on various media the analytical meterials were placed into GasPak jars (BBL) containing an atomosphere reduced in oxygen content.

All bacteriological analyses of water and sewage samples and identification of bacterial isolates was carried out by the field staff at the Dorset Microbiology Laboratory. Biotyping and serotyping of selected isolates of Y.e. was conducted at the Yersinia Reference Laboratory, Ontario Ministry of Health, Rexdale, Ontario, under the direction of Dr. S. Toma.

(a) Assessment of the method of Harvey et al (1976) for isolation of Yersinia enterocolitica from lake water and sediment.

Lake water and sediment samples were collected from various sites during October and November of 1977 and analysed by the procedure of Harvey et al (1976) for Y.e. Sediment samples were treated by thorough shaking followed by two successive centrifugations at 1500 rpm for 10 minutes using a Servall (Model RC2) automatic-refrigerated centrifuge with type GSA centrifuge head operated at 4°C. Sediment supernatants were aseptically collected following centrifugation and thereafter treated in the same manner as were water samples.

Sediment supernatants and surface waters were analysed on the same day that the samples were obtained using procedures very similar to those described for the recovery of Y.e. from lakes and streams by Harvey et

al (1976). From each sample 5 tubes of glucose broth were inoculated with 1 ml of samples.

In this study the formulation of glucose broth differed from that of Harvey et al (1976) in containing, per 1000 ml, 5.0 g each of soluble casein and peptone (Difco) instead of 10 g of "casein peptone (Difco)". The inoculated tubes of glucose broth were incubated anaerobically at 20°C and checked daily for evidence of acid production for up to 96 h. When acidification of this medium was observed 1-3 loopfuls of culture were transferred, by streaking, to the surface of a plate of MT80 which in turn was incubated first anaerobically for 48 h at 20°C and then for an additional day at room temperature under aerobic conditions. After the anaerobic incubation and after the subsequent aerobic incubation the MT80 plates were observed for colonies which were indicative of Y.e. To assist in recognizing Y.e.-like colonies on MT80 during the early stage of this study a pure culture of Y.e. was used to inoculate a positive control tube of glucose broth in each experiment and these tubes, once acidified, treated in the same manner as those used for sample analysis and causing acidification of the glucose medium. The study of Harvey et al (1976) implied that Y.e. colonies recovered on MT80 might appear as non-fermentative, lipolytic colonies.

b) Assessment of urea deoxycholate broth for the detection of Yersinia enterocolitica in lake water and sediment.

Pickett (1977) reported success in the detection of Y.e. in waters of mountain lakes by employing UDB as a selective-enrichment medium and several enteric agars as isolation media incubated at 30°C. Using a slightly different formulation of UDB and using MT80 as an isolation medium we made several attempts to recover Y.e. from surface waters and from supernatants of shoreline sediments of several lakes in Muskoka during 1978.

For each sample analysis 5 ml of sample was inoculated into each of 5 tubes of UDB which had previously been warmed to room temperature to help liquefy this semi-solid double strength medium. The contents of these tubes were then mixed by vortex and observed daily for up to 21 days for evidence of urease production indicated by alkalinization of the medium and colour change from orange to pink or deep-pink or red. Alkalinization of this medium occurs due to the release of ammonia through the catabolism of urea by bacterial urease. A loopful of culture from each urease-positive tube of UDB was then transferred by streaking onto a plate of MT80 to isolate various enteric bacteria. These plates were then incubated for 48 h at 25°C and selected colonies picked from these and restreaked as a saline water suspension onto fresh MT80 to purify each culture. After 48 h at 25°C, or after 24 h at 35°C, well isolated colonies were again selected and retained for screening and identification. Colony morphologies of each

selected isolate growing on MT80 at 25°C and/or at 35°C were recorded in detail to familiarize each observer with the colonial characteristics of colonies subsequently identified as Y.e.

During the initial experiments, conducted in April and June 1978, inoculated tubes of UDB were incubated at several temperatures (4, 10, 20, 30 and 35°C) both aerobically and anaerobically and plates of MT80 incubated 48 h at 20°C. Thereafter UDB tubes were always incubated aerobically at 20°C with isolation of urease-positive cultures carried out on MT80 incubated for 48 h at 25°C. Purification of isolates obtained on MT80 incubated at 25°C was achieved by restreaking saline-water suspensions of individual colonies onto MT80 or tryptic soy agar (TSA) which were in turn incubated for either 48 h at 25°C or for 24 h at 35°C. As previously described, positive controls were employed in each of the experiments.

An additional experiment was carried out using water isolates of Y.e. obtained from another study to be described below. In this experiment tubes of UDB were inoculated with 5 ml volumes of water suspensions of 6 strains of Y.e., cultures 421, 445, 455, 457, 459 and 460. Of these, only culture 445 was serotypable, as 0:6,30, and all 6 strains were biochemically similar to biotype 1 Y.e. In this experiment the UDB tubes inoculated with pure cultures of these strains were incubated aerobically at 4, 10, 20, 30 and 35°C to ascertain the rates at which positive urease reactions were obtained at different temperatures.

c) Isolation of Yersinia enterocolitica and Yersinia ruckeri from wildlife intestinal contents using cold-enrichment, UDB and MT80.

Wildlife undoubtedly are an important reservoir of Y.e. in the natural environment (Botzler et al 1968, 1976; Langford 1972). In order to obtain some information concerning the presence of Y.e. in fur-bearing animals common to aquatic environments of Ontario a brief study was initiated during the winter months of 1978-1979. In this study the carcasses of several trapped beavers (Castor canadensis) and a muskrat (Ondatra zibethica) were supplied to us by a local trapper, Mr. Orrie Avery. Immediately upon notification that carcasses were available samples of intestinal contents, usually weighing 10 g for beaver samples, 3 g for muskrat samples, were obtained by aseptic removal of portions of the lower intestinal tract of each carcass. A sample from each species was obtained on December 11, 1978 and from another beaver carcass early in 1979. Upon removal of these materials from the carcass the feces were placed into bottles containing 300 ml of sterile phosphate-buffered water, shaken thoroughly and then incubated at 4°C. At the start of this cold-enrichment incubation and at weekly or bi-weekly intervals thereafter loopfuls of each mixture were transferred directly, by streaking, to plates of MT80 and these incubated for 48 h at 25°C and then examined for the presence of Y.e.-like colonies. Also on each of these occassions, for up to 7 weeks, tubes of UDB were inoculated with each cold-enrichment mixture in the usual manner. On each of these occassions an equivalent volume of sterile buffered water was used to replace that used in UDB

analysis. For a period of up to 21 days the UDB tubes which displayed alkaline reactions were employed to inoculate plates of MT8O. These plates were incubated in the usual manner at 25°C and selected cultures restreaked to MT8O for purification then retained for identification. The cultures selected were those with Y.e.-like colony characteristics and a limited number of colonies which possessed other unique characteristics.

d) Development and Assessment of a Membrane Filtration Procedure for Yersinia enterocolitica.

During the course of another investigation which was concurrently being carried out to assess the membrane filtration procedure of Fliermans et al (1977) for the determination of Aeromonas hydrophila in lake water, Y.e. was serendipitously discovered among the background colonies on membrane filters used to analyse a water sample in April of 1978. From that sample a 25 ml volume of lake water yielded a total of 38 colonies, 6 of which were confirmed as Y.e. These Y.e. isolates appeared as small (0.2 - 0.6 mm diameter) convex, colorless to very pale yellow colonies with or without minute dark centres after 22 h incubation of the membrane filter at 35°C on RSB. This finding suggested the potential value of examining the membrane filtration procedure and RSB medium of Fliermans et al (1977) in greater detail as a possible means of both detecting and quantifying Y.e. in lake water. Because of commitments to other projects, however, little work could be done in these regards until the fall of

1978. Never-the-less, many membrane filters which had been used to analyse lake waters for Aeromonas hydrophila during the interim were closely examined for the small usually colorless Y.e.-like colonies that had been first observed in April. No further isolates of Y.e. were obtained with these procedures, however, until November 1978 even though numerous colorless colonies were detected but found to be members of other bacterial genera (eg. Enterobacter spp., Serratia spp.).

Late in 1978 several attempts were made to improve the recovery of Y.e. on membrane filters incubated on RSB. These attempts involved the additions of the antibiotic, cephalothin (courtesy of Eli-Lilly Canada Ltd.), at final concentrations of 10 and 20 micrograms per ml to RSB, the use of rinse water containing 0.1% (w/v) yeast extract during the membrane filtration process, and the use of anaerobic incubation conditions for membrane filters on RSB. These modifications of the normal membrane filtration procedure were found to provide no improvement in the incidence of detection of Y.e. compared to that of control membrane filter incubations and RSB that was not modified in any way.

However, in December of 1978, attempts were made to utilize the strong urease activity of most strains of Y.e. growing at 35°C (Nilehn 1967) as a means of more easily distinguishing Y.e. colonies among the background colonies of membrane filters incubated on RSB at 35°C. Several experiments were conducted initially by first incubating

membrane filters in the usual manner on RSB for 22 h at 35°C and then transferring the filters to a second pad of absorbant paper saturated with urease test reagent (Difco). This urease reagent did permit the rapid discrimination of urease positive colonies on membrane filters kept at 35°C for only a few minutes but, since it lacked a buffering system and was employed in liquid form, the alkalinization reaction spread throughout the membrane filter so quickly that most colonies quickly took on the red coloration of urease-positive colonies. For this reason urease test reagent was not considered for further detailed examination.

However, much success was achieved when filters were incubated on RSB in the usual manner and then transferred to plates of CUA and incubated at 35°C. Preliminary observations made of membrane filters used to analyse several different volumes of lake water samples in December 1978 and in January 1979 and exposed to a primary incubation on RSB at 35°C for 22 h and then to a secondary incubation on CUA at 35°C for 24-48 h revealed that Y.e. could be detected in nearly every sample analysed using these procedures.

In order to permit quantitation of Y.e.-like colonies on these membrane filters and to provide information that would permit the formulation of a specific target colony (Y.e.-like colony) or presumptive target colony description a detailed system of records was devised and applied to many analyses carried out in 1979 using procedures summarized in Figure 1. In order to permit recording of

presumptive target and presumptive background colony counts and morphological characteristics scaled maps of the gridded membrane filters were prepared and the position and relative size of each colony on selected filters recorded on these maps at the time of each observation of the filters. Although one or more of the membrane filters used to analyse various volumes of a particular sample and bearing up to 300 total colonies after secondary incubation were used to obtain counts of presumptive target colonies (PTC) and presumptive background colonies (PBC) those selected for mapping and detailed examination usually were those which bore between 10 and 50 colonies after primary incubation. Mapping and detailed study of filters with higher total colonies counts was not deemed practical. An example of the mapping and recording procedures as well as photographs of colonies recovered by the RSB-CUA procedure as well as by MT80 are presented in Appendix A.

The first observations of each filter being mapped were recorded after primary incubation of the filters on RSB. At this time the position of each colony observed was marked on a map and assigned a map number. Concurrently, data sheets were used to record in detail the morphologies of each numbered colony. Similarly, after these filters had been exposed to secondary incubation on CUA for 24, 48 or, in some cases, for 72 and 96 h, colony characteristics (size, colour, texture, border, elevation, urease reaction etc.) were recorded for all colonies first seen after primary incubation and for all new colonies which appeared during each successive 24 h interval of secondary

incubation. The position and relative size of each new colony was recorded on the appropriate scaled map. In this manner we became familiar with the changes in colony numbers and colony characteristics associated with incubation of the membrane filters for different periods on RSB and CUA.

Whenever possible all of the mapped colonies were retained for screening, identification, and for subsequent biotyping and serotyping characterization. Practical considerations, however, limited the numbers of mapped filters which could be studied fully. Never-the-less many of the PTC observed on mapped or non-mapped filters, and a similar number of PBC observed on these filters, were subsequently identified to species and/or characterized serologically. An example of the mapping and recording procedures used to study the development of colony morphologies on a membrane filter used to analyse lake water by the RSB-CUA procedure is attached as Appendix A.

Using the information gained from these preliminary mapping and recording procedures together with the results obtained from the identification of PTC and PBC we were able to reliably distinguish Y.e.-like colonies visually, and to prepare a definitive PTC description for use with the routine application of the RSB-CUA system to water analyses by early March 1979. For instance, we learned that most Y.e., if present, on membrane filters following primary incubation were usually very small (0.1 - 0.3 mm diameter), colorless or pale yellow or tan coloured, convex, and sometimes possessed a

minute dark central spot. The majority of PTC, however, were first observed only after the first 24 h period of secondary incubation and the diameter and relative distinctiveness of PTC varied with age of the observable PTC and with the numbers and types of adjacent PBC. Most PTC appeared as colorless, convex colonies with a diffuse pale blue or well defined pale blue or colorless slightly opaque central area. These colonies were usually 0.5 - 1.0 mm in diameter, and usually were urease positive after 24 h of secondary incubation. New PTC which first appeared only after 48 h of secondary incubation had a similar appearance but some, necessarily recorded as PBC, were colorless and devoid of distinctive characteristics. PTC which were observed after 24 h of secondary incubation, especially those which were already observable as colonies after primary incubation, and those which were well isolated, usually were distinct from all PBC after 48 h incubation on CUA by possessing one or more differentiating rings about the central part of the colony. Most of these "mature" PTC had a well defined blue, blue-green or blue-and-white-marbled centre surrounded by one or more usually darker blue ring(s) and possessed usually a slight, flat, clear, colorless border. With extended incubation the most mature PTC often developed a slightly concave centre or a slight circular depression about the coloured centre. These PTC were always urease-positive, slightly convex and 0.6 - 1.5 mm in diameter. Since strong urease producers or the presence of numerous urease-positive colonies on filters after 48 h secondary incubation may obscure the urease reaction beneath individual colonies we adopted the procedure of always observing and counting PTC at both 24 h, when the urease reaction is most discrete

below individual colonies, and at 48 h of secondary incubation. This PTC description and the RSB-CUA system were subsequently applied to the analyses of approximately 1,000 surface water and sewage samples during the period of February 1979 to January 1980. Several experiments were carried out concurrently to assess the specificity and selectivity of the system and to attempt to improve upon these characteristics of the system. The following presentation describes these experiments in which use of various regimes of incubation duration and temperature and use of additional inhibitors and antibiotic were assessed in influencing recoveries of PTC and PBC by the RSB-CUA system.

d) i) Influence of incubation temperature of RSB on recovery of Yersinia enterocolitica and background colonies.

Three separate experiments were conducted in April 1979 to determine the influence of primary incubation temperature of RSB on the recoveries of PTC and PBC. Water samples were analysed by membrane filtration (Figure 1) over the range of 0.1 to 100 ml with filters incubated on RSB at 20°C and 35°C in the first experiment, at 35°C and 39°C in the second, and at 35, 39, 41.5 and 44.5°C in the third experiment. Replicate analysis was conducted only in the first experiment and the duration of primary incubation was for 48 h for those filters kept at 20°C and for 22 h for filters kept at all other temperatures. All secondary incubations of filters were carried out for 48 h at

35°C. Also, in the first experiment only, subjective comparisons were made between morphologies, particularly the yellow coloration, of PBC recovered on CUA and on mCUAM. Selected colonies from each filter were retained and subjected to screening and identification tests (Figure 1).

ii) Influence of increased novobiocin concentration in RSB.

Because of the reported in vitro resistance of many strains of Y.e. to novobiocin (Raevuori et al 1978; Nilehn 1967), the antibiotic contained in RSB, 2 experiments were carried out to determine the influence of higher concentrations of novobiocin in RSB upon the recoveries of PTC and PBC using the RSB-CUA system. Membrane filtration of several volumes of samples obtained from a stream, from drainage water adjacent to a sewage lagoon as well as from a wastewater lagoon sample was carried out in the usual manner (Figure 1) in duplicate in May 1979. One set of filters was incubated on RSB (normal) containing 5 micrograms/ml novobiocin (Fliermans et al 1977) while the other set was incubated on RSB containing novobiocin at twice the normal concentration (10 micrograms/ml). Following primary incubation of these filters at 35°C for 22 h the total colony counts were recorded for each and then selected filters were transferred to CUA and exposed to secondary incubation in the normal manner. After secondary incubation (48 h) the PTC and PBC counts were recorded for each set of filters. Selected PTC from several filters were retained for identification.

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iii) Influence of increased concentrations of sodium deoxycholate in RSB.

Current literature concerning Y.e. attests to the usefulness of employing bile salts or deoxycholate in enteric media to improve the rate of detection of this bacterium (Mehlman et al 1978; Wauters 1973). The RSB used in this laboratory for the routine determinations of Aeromonas hydrophila, and for Y.e. (Figure 1), contained sodium deoxycholate at a final concentration of 0.1%. In order to determine if the apparent tolerance of Y.e. to deoxycholate could be used to improve the selectivity (reduce the relative PBC count) of the RSB-CUA system several experiments were carried out with lake water samples in April 1979 using normal RSB as well as RSB which had been modified by increasing the concentrations of sodium deoxycholate to 0.2% and to 0.5%. One problem which arose in the preparation and use of RSB containing sodium deoxycholate at concentrations of 0.2% or greater was that the medium became semi-solid at refrigeration temperature. For this reason it was necessary to warm these modified RSB preparations to 35°C just prior to use in order to liquefy them so that they could be dispensed to plates containing absorbant pads.

Duplicate membrane filtration and incubation procedures were performed in the usual manner for each experiment with 1 set of filters incubated on normal RSB and the other on RSB containing

0.2% in one experiment or a RSB containing 0.5% in the other experiment. Selected PTC and PBC were retained for screening and identification in each experiment.

iv) Comparison of broth and agar forms of Rimler-Shotts medium for the recovery of Yersinia enterocolitica.

The use of RSB in the primary incubation step of the RSB-CUA membrane filtration procedure for Y.e. inherently involves the carry-over of broth medium during the transfer of membrane filters to CUA for secondary incubation. In an effort to determine if the use of the agar form of Rimler-Shotts medium in the primary incubation step might reduce the recovery of PBC without simultaneously reducing the recoveries of PTC a simple experiment was carried out.

During the summer and fall of 1979 a total of 103 water samples collected during routine sampling surveys of lakes, rivers, and sewage systems in the study area were analysed in duplicate by membrane filtration. One set of filters from each sample was incubated in the usual manner on RSB during primary incubation (Figure 1) while the other set was exposed to primary incubation upon RSA. Secondary incubation and colony counting for all filters was conducted in the usual manner (Figure 1). From this experiment comparisons between recoveries of PTC and PBC were possible for a total of 103 sets of membrane filters used to

analyse 80 water samples using both RSB and RSA as the primary incubation media. Data from the analyses of the other 23 samples did not provide absolute values for PTC or PBC so were excluded from the comparison.

v) Recovery of Yersinia enterocolitica human isolate (serotype 0:3) from water suspension by membrane filtration.

Y.e. strains of serotype 0:3 account for most of the cases of human yersiniosis in eastern Canada (Toma, Lafleur and Deidrick 1979) but only recently has this serotype been isolated from water and non-animal sources (Dr. G. Kapperud, personal communication). In order to determine the capacity of the RSB-CUA system to recover Y.e. serotype 0:3 from water a brief experiment was conducted.

Using a human clinical isolate of Y.e. serotype 0:3 a water suspension of this strain was prepared by suspending a loopful of culture that had been maintained on a slant of TSA for two weeks in 90 ml of sterile phosphate-buffered water. After thorough shaking serial dilutions of this original suspension were made using 90 ml buffered dilution blanks, with thorough shaking of each successive dilution. The original suspension was diluted throughout the range of 10^{-1} to 10^{-9}. Each dilution blank in this series was kept refrigerated until required for analysis and reshaken vigorously just prior to analysis.

From each dilution bottle through the range of 10^{-3} to 10^{-9} a volume of 50 ml was analysed using the RSB-CUA system as described in Figure 1. Concurrently, from each dilution blank used for membrane filtration analysis 5 replicate plates of TSA were each inoculated by the spiral pipetting technique with 0.5 ml of culture suspension.

Membrane filters were then incubated and observed in the usual manner (Figure 1) and the TSA plates incubated at 35°C and observed daily. Colony counts on TSA plates were determined after 24, 48, and 72 h of incubation.

vi) Determination of the specificity and selectivity of the RSB-CUA system for Yersinia entercolitica.

In order to obtain estimates of the presumptive and confirmed specificity and selectivity of the RSB-CUA system in recovery of Y.e. from aquatic samples the data obtained during the study period of February 1979 to January 1980 was examined in detail and calculations of gross selectivity for the entire study period and for various types of samples and for various study intervals were made.

The gross presumptive selectivity index was estimated using the forumla:

$$\frac{\text{Cumulative Observed PTC}}{\text{Cumulative Observed PTC plus Cumulative PBC}} = \text{presumptive selectivity index}$$

In this calculation only data from membrane filter colony counts for surface and well water analyses where positive absolute values of either PTC or PBC were obtained were used. Data from wastewater analyses or from samples obtained from sewage lagoon systems and data for which only an estimate of a count per 100 ml of sample for either PTC or PBC was obtained were excluded from this calculation. Also excluded from this and other calculations of selectivity were results obtained from membrane filters on which more than 300 total colonies (PTC and PBC) were observed. Similar calculations of gross presumptive selectivity indices were also made for data collected in each month during the study period.

Because Y.e. was rarely detected during periods of warm surface water temperatures, when the greatest proportion of samples were analysed (Lautenschlager 1980), additional calculations of presumptive selectivity indices were also made. In these calculations, concerning only surface and well water analyses, only the results concerning membrane filters on which observed PTC counts of 1 or more colonies were obtained were included in the calculations. As described above, results of analyses yielding only estimates of either PTC or PBC were excluded from the calculations.

Calculations of confirmed selectivity indices for individual membrane filter analyses and for 12 membrane filters for which

all PTC and PBC were retained for identification, following surface water analyses by the RSB-CUA system, were also made. In these calculations the confirmed selectivity indices were calculated using the following formula:

$$\frac{\text{Total Confirmed Target colonies}}{\text{Total Confirmed target colonies + False PTC + Total confirmed background colonies}} = \text{confirmed selectivity index}$$

Similar calculations for the results of 3 samples from sewage lagoon systems were also made.

Confirmation of observed colonies as target or as background colonies was made using the API-20E biochemical test system. Where isolates of PTC or PBC died before confirmation they were considered to be confirmed target or background colonies, respectively, for purposes of these calculations. Loss of cultures due to death was particularly common among PBC retained for identification. Most losses occurred as a result of the isolates' failure to grow on MacConkey agar or upon MT80 and this was considered as indicative of non-Y.e. or as confirmed background colonies. In all instances the PTC cultures that were lost before confirmation possessed colony morphologies which were characteristic of mature PTC and therefore were considered as confirmed target colonies for purposes of these calculations.

The gross specificity of the RSB-CUA system for the determination of Y.e. in surface waters was estimated by the following procedures. The total number of distinct PTC which were recovered on membrane filters used to analyse 208 Y.e.-positive surface water samples and which survived to be identified by the API-20E biochemical system was determined. The proportion of these colonies which were confirmed as Y.e., expressed as a percentage of the total of successfully identified PTC, was considered to be an accurate estimate of the specificity of the RSB-CUA system when applied to surface water analyses over a 1 year period.

Efforts were also made concurrently with these calculations to determine the identification of false PTC and the colony characteristics of PBC which were subsequently identified as Y.e.

Chapter IV

Results and Discussion

a) Assessment of the method of Harvey et al (1976) for isolation of Yersinia enterocolitica from lake water and sediment.

In each of the two attempts to isolate Y.e. from lake water or sediment using procedures similar to those employed by Harvey et al (1976) several serious difficulties were encountered which subsequently led us to abandon these procedures.

Firstly, every tube of glucose broth which was inoculated with lakewater or sediment and incubated anaerobically at 20±2°C became acidified within 24 h thus necessitating the transfer of broth culture from every tube to plates of MT80. So rapid and intense was the fermention in each inoculated tube that the glucose was apparently completely utilized within 48 h and subsequent utilization of casein and/or peptone as a carbon source brought about a reversion of colour in most tubes from yellow to blue-purple. Failure to observe the glucose broth tubes before such reversion occurs could mean that the acid reaction from glucose fermentation could be overlooked in some instances.

Secondly, transfer of the recommended 3 loopfuls of inoculum from acidified glucose broth tubes to plates of MT80 always resulted in excessive growth of bacteria on the agar plates making discrimination

of individual colonies impossible and masking any reactions indicative of lactose fermentation or lipolysis of Tween 80.

Thirdly, when only 1 loopful of broth culture was transferred to plates of MT80 isolated colonies were obtained by streaking but these too were very numerous and differed in morphology, and in lactose and lipolysis reactions so that it was deemed unlikely that Y.e. would be detected unless excessive numbers of colonies be chosen and identified. Since the positive control broth cultures yielded non-lipolytic, lactose-negative colonies on MT80 there were no colony characteristics available to us at that time which would permit us to differentiate between possible Y.e. colonies and the numerous other colony types on the MT80 plates.

As such this method did not appear to afford us a practical means by which water or sediment samples could be routinely analysed for Y.e. with any reasonable degree of success in isolating this bacterium.

Although we chose to abandon this method in favour of other procedures to be described below, Harvey et al (1976) were successful using similar procedures in isolating 10 strains of Y.e. from mountain lakes and streams in remote parts of California during the summer months. Most (9) of these isolates were nontypable by the serological typing systems available at that time.

b) Assessment of urea deoxycholate broth for the detection of Yersinia enterocolitica in lake water.

The results of one experiment to assess urea deoxycholate broth (UDB) as a selective-enrichment medium for detection of Y.e. in lake water are shown in Tables 1 and 2. By incubating tubes of UDB inoculated with a lake water sample of April 4, 1978 or with a pure culture of Y.e. and incubating these tubes at various temperatures under aerobic and anaerobic conditions it was found that Y.e. was most frequently isolated following incubation of UDB tubes under aerobic conditions at 20 and 30°C (Table 1). Seven of the eight isolates of Y.e. obtained from the lake water sample were from aerobic incubation of UDB at 20 and 30°C while only 1 isolate was obtained from a tube of UDB incubated anaerobically at 20°C. Isolates of Y.e. were not obtained from those tubes of UDB incubated at 3, 10 or 35°C. Also, the stock culture of Y.e. did not cause alkalinization of UDB when incubated at 4°C for up to 21 days.

The effect of incubation temperature on urease activity of Y.e. in UDB is more evident from the results shown in Table 2. In all but one instance (culture 457 at 10°C) the 6 water isolates displayed greater urease activity at the higher incubation temperatures, 20-35°C. Variation between the 6 strains in bringing about the alkalinization of UDB medium was apparent at each incubation temperature but was more pronounced at the two lower temperatures.

Table 1

Periods required for alkalinization of urea deoxycholate broth (UDB) tubes inoculated with lake water or with Yersinia enterocolitica (Y.e.) and numbers of UDB tubes yielding Y.e. after incubation at various temperatures under aerobic or anaerobic conditions.

Source of Inoculum for UDB Tubes	Incubation Temp. (°C)	Aerobic		Anaerobic	
		Number of UDB Tubes Yielding Y.e.	Period Required for alkalinization of 1 or more UDB tubes	Number of UDB tubes yielding Y.e.	Period required for alkalinization of 1 or more UDB tubes
Lake Water Sample of April 4, 1978	4	0	*	0	N.C.
	10	0	*	0	N.C.
	20	4	9 days	1	14 days
	30	3	4 days	0	N.C.
	35	0	N.C.	0	N.C.
Y.E.[x] Stock Culture (positive control)	4	N.D.	N.C.	N.D.	N.C.
	10	N.D.	8 days	N.D.	N.C.
	20	1	8 days	N.D.	14 days
	30	1	10 days	1	17 days
	35	1	8 days	1	16 days

* = alkaline UDB tubes were observed after 17 to 21 days but did not yield Y.e. in MT80.
N.C. = no change in any UDB tube observed during 21 days incubation.
N.D. = not determined.
x = only 1 tube of UDB was inoculated with Y.e. as a positive control for each treatment.

Table 2

Minimum periods required for six water isolates of Yersinia enterocolitica to produce alkalinization of urea deoxycholate broth (UDB) tubes incubated aerobically at various temperatures.

Incubation Temp. (°C)	Period required (days) for various Y.e. strains to produce alkalinization of at least 1 tube of UDB.					
	*421	445	455	457	459	460
4	7	14	14	4	7	17
10	7	11	7	7	7	11
20	3	8	2	2	3	7
30	3	7	2	3	3	7
35	2	2	1	2	1	7

* Y.e. culture collection numbers.

These observations are in agreement with previous reports concerning the effects of incubation temperature upon the expression of urease activity of Y.e. grown in laboratory media (Nilehn 1967, 1969b).

The bacterial colonies which appeared on MT80 plates inoculated with 1 loopful of culture from urease - positive tubes of UDB were generally quite diverse in their morphological characteristics and displayed a variety of reactions with regard to lactose fermentation and lipolysis. However, with experience gained with the picking and identifying of many colony types it was observed that on MT80 incubated at 25°C for 48 h most discrete colonies of Y.e. possessed the feature of having a slightly convex and/or rough or granular

centre. In general, Y.e. formed relatively small (0.5 - 1.5 mm) glossy colonies on MT80 at 25°C after 48 h and these usually possessed an irregular border and were usually surrounded by a halo of precipitate characteristic of lipolytic bacteria. Coloration of these colonies varied from translucent white (non-fermentative) to opaque pink or deep pink in response to the individual colony's capacity to utilize lactose in the agar medium. In supplementary tests it was learned that when grown or recovered on MT80 incubated at 35°C Y.e. colonies very rarely displayed the concave or granular centre or lipolysis which were features of much value in helping to discriminate between colonies of Y.e.-like organisms and other bacteria or MT80 incubated at 25°C. Incubation of MT80 at 25°C, therefore, was considered to be a procedure which markedly reduced the effort required in detection of Y.e. from environmental and lake water samples. Photographs of Y.e. colonies grown on MT80 at 25°C are presented in Appendix A.

These growth characteristics of Y.e. on MT80 at 25°C were later utilized to detect Y.e. in sewage influents by direct plating of as little as 10^{-4} ml of influent onto MT80. A future report (in preparation) will address this plating procedure for sewage analysis in greater detail.

Following the analyses of the lake water sample of April 4, 1978 (Table 1) an additional 9 surface water and shoreline sediment samples were analysed using UDB incubated aerobically at 25°C. From these

samples, obtained during the period of June 5 to November 15, 1978, Y.e. were not isolated. However, during the analyses of these samples a total of 34 of 50 inoculated UDB tubes were observed to be positive for urease activity during the 21 day enrichment incubation. Two of the 10 samples did not produce any urease-positive UDB tubes. From the 34 urease-positive UDB tubes a total of 68 discrete colonies were picked from MT80 isolation plates even though none of those selected for identification possessed Y.e.-like colony characteristics. Of these cultures 14 were identified as Serratia spp., 12 as Aeromonas hydrophila, 7 as Enterobacter spp, 4 as Pseudomonas spp., 3 as Citrobacter freundii; 1 as Proteus morganii, 1 as Proteus vulgaris and 1 as Moraxella spp. Nine cultures died before they could be identified and 16 could not be identified to genus or species using the API-20E biochemical test system. Members of these genera are apparently commonly encountered in fresh waters (Shotts and Rimler 1973; Dudley and Shotts 1979) and therefore can be expected to be recovered on enteric media. However, not all of the species recovered from urease-positive UDB tubes are known to produce urease so the observed alkalinization of UDB tubes may in some instances have been due to organisms other than those recovered on MT80 or due to the effects of numerous weak producers of urease. None of these, when present as discrete colonies on MT80, incubated at 25°C, however, formed colonies with concave or rough centres which was characteristic of most observed isolates of Y.e. Therefore, when present as well isolated colonies on MT80 after incubation for 48 h at 25°C they afforded little problem in being visually distinguished as non-Y.e. colonies.

Using UDB of a similar formulation as a selective enrichment medium, but incubated at 30°C, together with several enteric isolation agar media incubated at 30°C the working group of Pickett (1977) isolated 34 strains of Y.e. from 110 water samples obtained from the Mammoth Lakes area of California. In the present study 8 strains of Y.e. were isolated from 1 lake water sample collected in April of 1978 (Table 1). Of these isolates 2 were serotypable as 0:17 and 0:16,29 while the remainder were nontypable by current serological typing procedures. Y.e. serotype 0:17 has been involved in a variety of human infections (Bottone et al 1974; Bottone 1977, 1978) and a water isolate of this serotype has been shown to be pathogenic to chinchilla in laboratory pathogenicity tests (Raevuori et al 1979).

Although the use of UDB enrichment and MT80 isolation media did provide a means of detecting Y.e. in lake water samples obtained during winter months its application to water analysis on a routine basis is impractical. Since Y.e. was rarely detected by other apparently more reliable and quantitative procedures during periods when lake water temperatures of the study area exceeded 10-15°C (Lautenschlager 1980) the use of the UDB-MT80 enrichment procedure for lake water analysis during summer months is not justifiable. Its application to detection of Y.e. in feces and other particulate materials, however, may deserve further investigation.

c) Isolation of Yersinia enterocolitica and Yersinia ruckeri from wildlife intestinal contents using cold-enrichment, UDB and MT80.

Direct inoculation of phosphate buffered water suspensions of the intestinal contents of beaver and muskrat obtained on December 11, 1978 onto MT80 yielded no colonies with the appearance of those formed most commonly by Y.e. grown at 25°C for 48 h. However, 2 strongly fermentative colonies derived from the beaver sample were identified as Escherichia coli and 1 small non-fermentative colony of the same origin was tentatively identified as Acinetobacter sp. From 4 colonies derived from the muskrat sample 1 was identified as Enterobacter cloacae, 2 as Acinetobacter sp. and 1 died before identification. Escherichia coli-like colonies, which dominated the types of colonies recovered on MT80 from beaver were not observed on MT80 inoculated with fecal suspension from muskrat.

Of the 5 tubes of UDB which were inoculated with the freshly prepared water suspension of intestinal contents of beaver on December 11, 1978 3 tubes displayed evidence of urease activity after 6 days of incubation. Culture from each of these 3 tubes was transferred to MT80 while the other 2 tubes were discarded without further treatment. From the inoculated plates of MT80 no Y.e. cultures were obtained but 3 isolates of Escherichia coli, from each tube, and two cultures of Enterobacter hafniae, were obtained. Y.e.-like colonies were not observed on these plates.

All 5 UDB tubes inoculated with freshly prepared suspension of contents of muskrat intestine were found to be strongly urease-positive when observed after 23 days incubation at 20°C. These tubes likely were alkaline prior to this time but were not observed earlier

because of the closure of the laboratory during the annual winter vacation period. Due to other commitments, however, only 2 tubes were further examined. From these tubes no Y.e.-like colonies were recovered on MT80 but one strain each of Serratia liquefaciens and Yersinia ruckeri were isolated. This strain of Yersinia ruckeri appeared as a smooth, glossy, weakly- fermentative, lipolytic colony, about 2 mm in diameter with a slightly irregular border on MT80 after 48 h at 25°C. In pure culture on MT80 incubated at 35°C for 48 h Yersinia ruckeri formed greyish-white nonfermentative, nonlipolytic colonies which were slightly convex, transparent, smooth with a dull gloss and mostly about 2 mm in diameter. To our knowledge this strain of Yersinia ruckeri is the first reported enteric isolate of this organism to be derived from an animal other than fish. In subsequent pathogenicity tests this strain was shown to be highly virulent and lethal to trout (James Daly, University of Guelph, personal communication).

UDB enrichment of contents of beaver intestine which had been incubated in buffered water at 4°C for 42 days produced positive results for urease activity in all inoculated UDB tubes in only 6 days at 20°C. From plates of MT80 inoculated with culture from each of these UDB tubes numerous Y.e.-like colonies were observed after incubation for 48 h at 25°C. A photograph of Y.e. colonies isolated from beaver feces on MT80 is presented in Appendix A (Figure 5a). Only a small number of Y.e.-like colonies and other colony types were picked for further study. Five colonies which appeared as Y.e.-like were subsequently confirmed as Y.e. All 5 of these were

rhamnose-fermenting strains of serotype O:21, biotype 1, Y.e. (Dr. S. Toma, personal communication). Among the other colony types examined isolates of Serratia spp., Enterobacter hafniae and Pseudomonas sp. were obtained. From the buffered water suspension of contents of muskrat intestine treated in the same manner no isolations of Y.e. were made and of the few other colony types examined all were identified as members of the genus Serratia. Application of these procedures to intestinal contents of a second beaver carcass in January 1979 did not yield any further isolates of Y.e.

Direct inoculation onto enteric media, selective enrichment, and cold-enrichment followed by isolation on various enteric media, have been widely used for the detection of Y.e. in fecal material from humans and wildlife, and from foods (e.g. Juhlin and Ericson 1961; Nilehn and Sjostrom 1967; Langford 1972; Otsuki et al 1973; Wauters 1973; Zen-yoji et al 1974; Eiss 1975; Greenwood et al 1975; Inoue and Kurose 1975; Toma and Deidrick 1975; Tsubokura et al 1975; Morris and Feeley 1976; Christenson and Jansen 1977; Hanna et al 1977; Lee 1977; Burton and Cody 1978; Mehlman et al 1978; Schiemann and Toma 1978; Sorger et al 1978; Thibault and Duval 1978; Dudley and Shotts 1979; Pai et al 1979; Pedersen 1979; Pedersen and Winblad 1979; Schiemann 1979; Stanek et al 1979; Stern and Pierson 1979; Aulisio et al 1980; Christensen 1980; Lee et al 1980; Soltesz et al 1980). Cold-enrichment of these materials is thought to provide conditions which are advantageous for the growth of Y.e. Apparently many strains of Y.e. are capable of utilizing nutrients from feces or foods to permit their growth under cold conditions to levels which make Y.e.

detectable even in the presence of numerous other enteric bacteria mostly of species which mask the presence of Y.e. in the sample initially. Used in conjunction with one or more selective enteric media incubated at temperatures between 22 and 30°C (Chester and Stotzky 1976) cold-enrichment provides the best means to date for detecting Y.e.-like bacteria in fecal specimens and in foods. In the present study Y.e. was isolated from fecal material taken from the lower intestinal tract of a beaver only after prolonged incubation of the material in buffered water at 4°C, whereas direct inoculation of an aqueous suspension of the material onto MT80 or after direct inoculation of the same suspension into UDB yielded negative results for Y.e. These results attest to the usefulness of a cold-enrichment procedure in bacteriological surveys of fecal material for Y.e. Since Castor canadensis defecates almost exclusively in the waters which it inhabits beaver feces may be considered to be a source of Y.e. as well as other bacteria in surface waters. How significant this source is to the aquatic population of Y.e. is unknown, however, since the isolates of this organism obtained from beaver in the present study may have been present in the intestinal tract in low numbers associated with the food or water consumed by the beaver examined and not necessarily as a member of the animal's intestinal microflora (Dr. M. Hacking, personal communication). Y.e. has been associated with disease in beaver in Ontario (Hacking and Sileo 1974).

The serendipitous discovery of a strain of Yersinia ruckeri among the bacteria isolated from the intestinal contents of muskrat suggests an

animal enteric reservoir of this important pathogen of salmonid fishes (Ewing et al 1978) which had not previously been recognized.

The failure to recover Y.e. from the intestinal contents of 3 animals by direct inoculation of this material onto MT80 suggests that this protocol is unlikely to permit isolation of Y.e. which is present in relatively low numbers in the presence of numerous other enteric bacteria. While direct inoculation of clinical fecal specimen onto enteric isolation media is considered to provide an adequate means for detecting Y.e. in symptomatic patients, cold-enrichment of such samples is recommended in cases of asymptomatic carriers and convalescing patients (Pai et al 1979). Therefore, in the study of the distribution of Y.e. in apparently healthy animal populations, particularly when carrier rates or densities of Y.e. in feces are expected to be low, the cold-enrichment procedure supplemented with selective-enrichment and/or direct plating of enriched sample onto various enteric media affords the best means of detection of this bacterium at the present time. Until a more selective isolation medium, specific for Y.e., is developed the prolonged incubation periods involved in the cold-enrichment procedure will continue to remain an inherent disadvantage in the determination of the sources and vectors of this bacterium.

d) Development and assessment of a membrane filtration procedure for Yersinia enterocolitica.

From February 1979 to January 1980 about 1,000 surface, well, and waste water samples were analysed using the RSB-CUA system schematically represented in Figure 1. The findings of these analyses for the period February 1979 to August 1979 have been reported elsewhere (Lautenschlager 1980). These findings indicated that the RSB-CUA system applied to water analysis provided a means of quantifying levels of contamination of surface and waste waters with Y.e. They also demonstrated that the RSB-CUA system recovered many different strains of serotypable and non-typable Y.e. including strains which were serologically and biochemically identical to strains of Y.e. involved in human yersiniosis in Canada or in diseases of wildlife. Although some of the short-comings of this membrane filtration procedure will be discussed in detail below, the application of the RSB-CUA system affords the first practical analytical technique for the determination of Y.e. and related species in aquatic environmental samples. As will be shown below, its use within the confines of the PTC description and in the hands of experienced technicians provides the means of determining Y.e. and Y.e.-like bacterial densities with a high degree of specificity.

i) Influence of incubation temperature of RSB on recovery of Yersinia enterocolitica and background colonies.

The results of the 3 experiments in which lake water samples were analysed using the RSB-CUA system (Figure 1) and sets of membrane filters incubated at various temperatures on RSB for primary incubation are shown in Table 3.

Figure 1 Flow chart of procedures employed for the isolation, identification, and characterization of *Yersinia enterocolitica* from water.

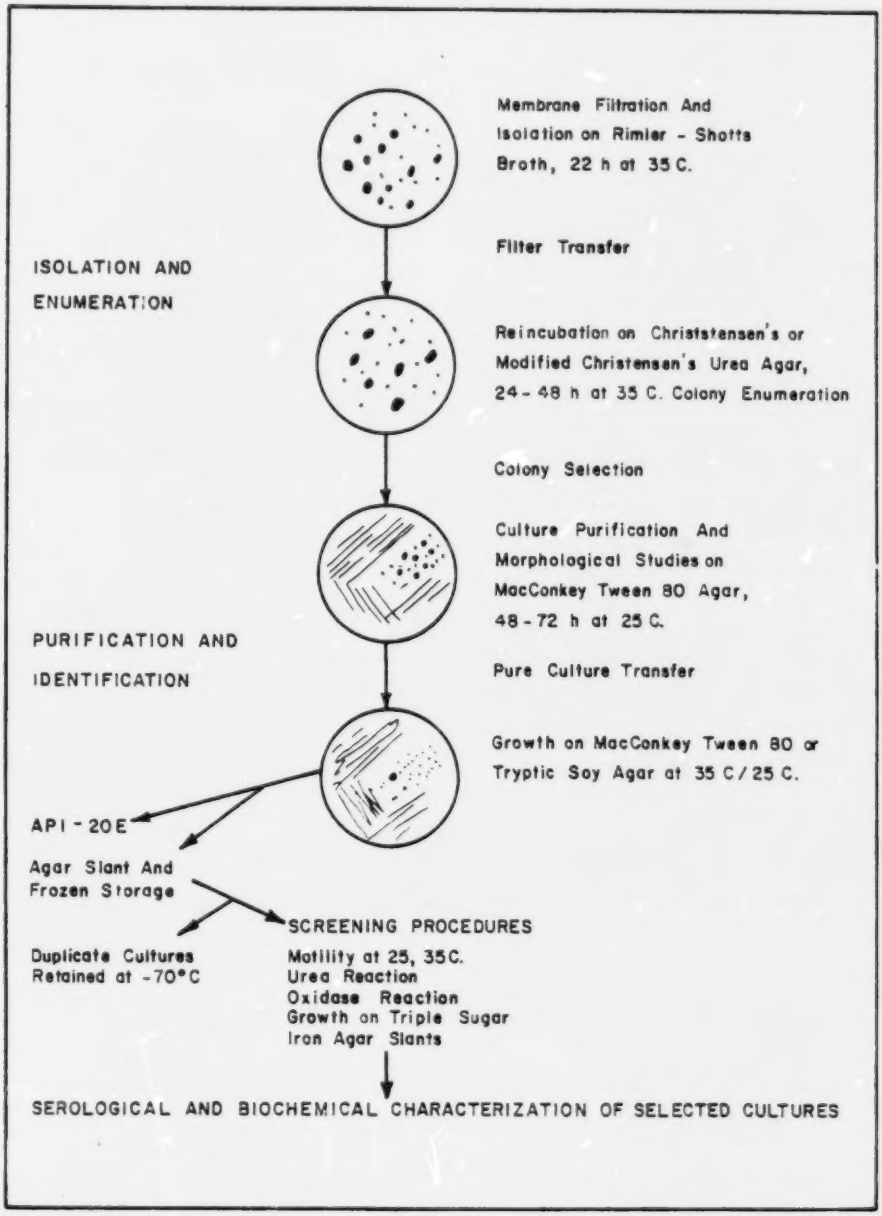

Table 3

Results of 3 experiments concerning effects of primary incubation temperature and duration on recoveries of PTC and PBC on urea agar after secondary incubation.

Experiment No.	Primary Incubation Regime	Sample Volume Analysed	Observed Colony Counts after 2^{o} Incubation		
			PTC	PBC	Total
1	48 h at $20^{o}C$	0.1	0	17	17
		0.1	0	9	9
		0.1[x]	0	10	20
		0.1[x]	0	12	12
		1.0	1*	TNTC	TNTC
		1.0[x]	2*	TNTC	TNTC
	22 h at $35^{o}C$	10	4*	26	30
		10	4*	23	27
2	22 h at $35^{o}C$	10	2	9	11
		25	5*	52	57
		50	22	97	119
	22 h at $39^{o}C$	10	1*	9	10
		25	2*	32*	34
		50	7	41	48
3	22 h at $35^{o}C$	25	4*	26	30
	22 h at $39^{o}C$	25	0	17	17
		50	3*	36	39
	22 h at $41.5^{o}C$	50	3*	29	32
	22 h at $44.5^{o}C$	25	0	11	11
		50	0	42	42

* at least 1 colony of PTC or PBC confirmed as Y.e.
x secondary isolation medium employed in these analyses was mCUAM
\# 2^{o} = Secondary

In the first experiment, in addition to the procedures already described, another set of membrane filters used for analyses were treated by secondary incubation on mCUAM in addition to those incubated by the normal procedure on CUA. The results of this experiment indicated that the extended primary incubation (48 h) of filters at 20°C resulted in an excessive increase in the recovery of PBC after secondary incubation compared to those filters treated in the normal manner (Figure 1).

Y.e. were, however, isolated from both sets of filters although those filters treated by primary incubation at 20°C possessed PBC populations which rendered the filters useless for counting purposes. The use of mCUAM did not provide any apparent advantage in these regards over the use of CUA as the secondary incubation medium although some organisms such as A. hydrophila did retain their strong yellow coloration (Shotts and Rimler 1973) for longer periods on mCUAM than on CUA. This apparently was the result of availability of maltose in mCUAM for which maltose fermenting colonies present on filters incubated on RSB would be well adapted to utilize as a carbon source.

The results of the second experiment indicated that Y.e. could be recovered from both sets of filters following primary incubation at 35°C or 39°C. The recovery of PTC was slightly better for filters incubated at 35°C but incubation of filters on RSB at 39°C resulted in lowered counts of PBC than were observed for filters used to analyse the same volumes of sample and incubated at 35°C.

The third experiment clearly shows that for a specific volume of water analysed (e.g. 25 ml) the observed counts of PBC decreased with increased temperature of primary incubation of membrane filters on RSB. In this experiment PTC and confirmed Y.e. were isolated from filters incubated on RSB at 35, 39 and 41.5°C but were not observed on filters incubated on RSB at 44.5°C. The detection of Y.e. on media incubated at 39°C or at higher temperatures is in agreement with the findings of Mehlman et al (1978) but conflicts with the observations of Maruyama (1973). The use of elevated incubation temperatures, including 35°C, to reduce levels of background colonies in an effort to improve the recovery of Y.e., however, may result in the selection of isolates of Y.e. which may be of little significance to public health. Many physiological characteristics including enterotoxin production (Feeley et al 1979), motility (Nilehn 1969a), biochemical reactions (Nilehn 1969b; Chester & Stotsky 1976; Bottone 1977) and the production of factors associated with virulence of certain strains of Y.e. (e.g. Gemski et al 1980; Maclagan & Old 1980; Zink et al 1980) may be influenced by the temperature and/or composition the medium on which cultures of Y.e. are grown. In some instances cultivation of some strains at elevated temperatures (e.g. above 32°C) may result in irreversible loss of some characteristics (e.g. serotyping antigens) which might be very important to the determination of the involvement of a particular strain in human or animal disease. Efforts should therefore be made to reduce the exposure of Y.e. isolates to temperatures above 30-32°C to a minimum

(e.g. no more than 24 h). In regard to the RSB-CUA system this suggests the need to improve the selectivity of the media by chemical means in order to permit incubation temperatures to be reduced without a concurrent increase in levels of recovered PBC.

ii) Influence of increased novobiocin concentration in RSB

The results of the experiment in which RSB containing the antibiotic novobiocin at concentrations of 5 (normal) and 10 micrograms/ml was used for primary incubation of membrane filters used for water and wastewater analyses are shown in Table 4.

No apparent difference in recoveries of PTC or PBC were noted for the filters used to analyse 1.0 ml of stream water and treated by primary incubation on normal RSB or on RSB containing 10 micrograms/ml novobiocin. In the examination of wastewater, however, the increased novobiocin concentration was apparently responsible for an observed reduction in observed PBC but the incidence of detection of PTC on these filters incubated on RSB containing 10 mg novobiocin/ml was also reduced compared to those results obtained for analyses using normal RSB. For instance, PBC counts on filters incubated on RSB containing 10 mg/ml novobiocin were generally 2-3 times less than PBC observed on corresponding filters incubated on normal RSB. PTC were detected on 5 of 8 filters used to analyse lagoon drainage or wastewater samples and given primary incubation on normal RSB but were

Table 4

Observed counts of presumptive target (PTC) and presumptive background (PBC) colonies on membrane filters (MF) used to analyse water and wastewater samples by the RSB-CUA procedure employing Rimler-Shotts broth (RSB) containing different concentrations of novobiocin.

Sample	Volume Analysed (ml)	Concentration of Novobiocin in RSB (micrograms per ml)							
		5 mg/ml (Normal)				10 mg/ml			
		MF Total Colony Count on RSB After Primary Incubation	MF Colony Counts on Urea Agar			MF Total Colony Count on RSB After Primary Incubation	MF Colony Counts on Urea Agar		
			PTC	PBC	Total		PTC	PBC	Total
Stream[1] Water	1.0	11	3*	11	14	12	2	12	14
Lagoon[2] Wastewater	10^{-3}	33	0	36	36	18	1*	19	20
Drainage[3] of Lagoon	10^{-2}	23	5*	21	26	3	0	9	9
Lagoon[4] Wastewater	1.0	182	2*	185	187	36	3*	46	49
	10^{-1}	10	2	17	19	1	0	6	6
	10^{-2}	2	1	2	3	1	0	1	1
	10^{-3}	2	0	5	5	0	0	0	0
Lagoon[5] Wastewater	10^{-2}	57	4	143	147	39	1	66	67
	10^{-3}	6	0	6	6	4	0	6	6

1. Obtained May 14, 1979 at Mary Lake
2. Obtained May 14, 1979 from Upper Cell of Ontario Pioneer Junior Camp
3. Obtained May 14, 1979 from drainage adjacent to source of Sample 2
4. Obtained May 22, 1979 from Cell No. 1, Pinestone Inn Lagoon System
5. Obtained May 22, 1979 from Cell No. 2, Pinestone Inn Lagoon System

* Indicates at least one isolate confirmed as Y. enterocolitica

observed on only 3 of 8 of the membrane filters incubated on RSB containing the higher concentration of novobiocin. These findings suggest that increasing the novobiocin concentration of RSB for use in the RSB-CUA system does not improve the relative selectivity of the RSB-CUA system for determination of Y.e.

However, in 2 instances (samples 4, 5, Table 4) the increased novobiocin concentration in RSB apparently resulted in a significant reduction in PBC without causing complete loss of PTC in two wastewater sample analyses. Further study should be done in these regards to determine the effects of increased novobiocin or other antibiotics which are not inhibitory to most strains of Y.e. (Raevuori et al 1978; Cornelius 1975) but which inhibit the more common PBC recovered from surface waters and wastewaters with the RSB-CUA system. At present no single antibiotic appears to be available which would inhibit strains of species of Aeromonas, Serratia, Klebsiella, Pseudomonas, Enterobacter and Acinetobacter, the background organisms most often observed in analyses with the RSB-CUA system, without inhibiting most strains of Y.e.

iii) Influence of increased concentrations of sodium deoxycholate in RSB

The results of the 2 experiments in which RSB was employed with normal or increased levels of sodium deoxycholate are presented

Table 5

Observed counts of total and of presumptive target (PTC) and presumptive background (PBC) colonies on membrane filters used for surface water analysis employing Rimler-Shotts broth (RSB) containing various concentrations of sodium deoxycholate for primary incubation and Christenson's urea agar (CUA) for secondary incubation

Sample	Value Analysed (ml)	Concentrations of sodium deoxycholate in RSB (%, W/V final concentration)											
		0.1 % (normal)				0.2 %				0.5 %			
		Total Colony Count on RSB	Colony Counts on CUA			Total Colony Count on RSB	Colony Counts on CUA			Total Colony Count on RSB	Colony Counts on CUA		
			PTC	PBC	Total		PTC	PBC	Total		PTC	PBC	Total
#1	10	4	2	5	7	N.D.	N.D.	N.D.	N.D.	0	0	0	0
	10	4	1	5	6					0	0	0	0
	25	7	1	24	25	N.D.	N.D.	N.D.	N.D.	0	0	2	2
	25	6	6*	30	36					0	0	6	6
	50	29	9	70	79	N.D.	N.D.	N.D.	N.D.	4	1*	15	16
	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	5	2	10	12
#2	10	6	1	11	12	0	0	0	0	N.D.	N.D.	N.D.	N.D.
	10	0	0	3	3	0	0	0	0				
	25	9	3	11	14	0	0	3	3	N.D.	N.D.	N.D.	N.D.
	25	14	0	17	17	3	0	4	4				
	50	21	10	31	41	2	0	0	10	N.D.	N.D.	N.D.	N.D.
	50	23	14	31	79	1	0	0	9				
	100	N.D.	N.D.	N.D.	N.D.	9	2*	13	15	N.D.	N.D.	N.D.	N.D.
	100					8	2	12	14				

Both samples (1,2) were obtained from Paint Lake, Station 1, on April 23 and 24, 1979, respectively
* Indicates at least one isolate confirmed as Y. enterocolitica
N.D. = not determined

in Table 5. In the first experiment it was observed that the use of sodium deoxycholate at a concentration of 0.5% markedly reduced the observed counts of PBC compared with results obtained using the usual analytical procedure (Figure 1) which employed RSB containing deoxycholate at a concentration of 0.1%. PTC were observed on each membrane filter exposed to primary incubation on normal RSB but were observed only on those filters incubated on RSB containing deoxycholate at 0.5% concentration for which the greatest volumes (50 and 100 ml) of samples had been analysed. For purposes of detecting and enumerating PTC with the RSB-CUA system the use of RSB containing deoxycholate at a concentration of 0.5% was not advantageous since it appeared to completely inhibit the growth of PTC and PBC in several instances where the sample volumes analysed (e.g. 10 ml) were small.

In the second experiment in which normal RSB and RSB containing 0.2% sodium deoxycholate were used for primary incubation of duplicate membrane filters PTC were observed on all filters incubated on normal RSB but only on 3 of the 6 filters incubated on RSB containing 0.2% deoxycholate. As in the first experiment the increased concentration of deoxycholate was apparently responsible for a significant reduction of observed PBC compared to the results for filters incubated on normal RSB. The 50 ml volume analyses, for instance, resulted in the recoveries of 10 and 14 PTC with PBC, respectively, of 41 and 79 on duplicate filters incubated on normal RSB. The duplicate filters used to

analyse 50 ml of the same sample but incubated on RSB containing 0.2% sodium deoxycholate yielded 2 and 1 PTC and 10 and 9 PBC, respectively. This suggests an approximate 10 fold decrease in recovery of PTC and a 4-8 fold decrease in recovery of PBC on filters exposed to primary incubation on RSB containing 0.2% deoxycholate. Although human isolates of Y.e. serotype O:3 are apparently very tolerant of even higher concentrations of deoxycholate than were used in this study (Wauters 1973) we did not observe an improvement in the gross selectivity of the RSB-CUA system by increasing the inhibitor concentration above 0.1%, the concentration at which it is employed in RS media (Shotts and Rimler 1973).

As previously mentioned, the use of sodium deoxycholate in RSB at concentrations of 0.2% or greater had the dissatisfactory characteristic of rendering the broth medium semi-solid at refrigeration and room temperatures. This necessitated warming these preparations of RSB to 35°C in order to permit them to be dispensed for use in membrane filtration analysis. The use of increased concentrations of deoxycholate in RSB in the membrane filtration procedure for water analysis therefore is not recommended under the conditions examined here. Further study should be done, however, to determine the effects of increased inhibitor concentrations in RSB, or by adding inhibitor to CUA, for applications of the the RSB-CUA system in which lower incubation temperatures (i.e. 25-30°C) are utilized.

iv) Comparison of broth and agar forms of Rimler-Shotts medium for the recovery of Yersinia enterocolitica

The results of membrane filtration analyses of 80 water samples using RSB and RSA for primary incubation of 103 duplicate membrane filters are shown in Table 6. PTC were observed on only 5 membrane filters incubated on RSB and were not observed on those incubated on RSA. Of the 7 PTC observed 5 were eventually confirmed as Y.e. An additional 3 isolates of Y.e. were obtained among selected PBC which appeared as clear, colorless colonies of 0.4-0.7 mm diameter on filters incubated on RSB. Of the PBC selected from filters incubated on RSA none were identified as Y.e. The totals for observed PBC for both sets of filters indicated a tendency towards a slight reduction in observed counts of PBC on those filters incubated on RSA compared to those exposed to primary incubation in the usual manner on RSB. However, for individual membrane filters the observed PBC counts on filters incubated on RSA were frequently greater than those observed on the corresponding filters analysed by the normal procedures using RSB as the primary incubation medium.

It should also be noted that on filters exposed to primary incubation on RSA the observed colonies of Aeromonas hydrophila among the PBC were generally much more distinct in their colonial morphologies after both primary and secondary incubation compared to A. hydrophila colonies observed on filters treated in the

usual manner. These observations led to the adoption of RSA in place of RSB for the determination of A. hydrophila in water. A report on these findings is currently in preparation.

It was also noted that most PBC colonies which were characteristic of species of Enterobacter, Serratia and Citrobacter (see Shotts and Rimler 1973) usually possessed a less distinguished coloration after secondary incubation for those filters incubated initially on RSA. This may have been due to the fact that the transfer of membrane filters from RSA to CUA did not involve the carry-over of nutrients and indicator which occurred in the transfer of filters from RSB to CUA. As will be discussed below the carry over of nutrients and indicator with membrane filters incubated on RSB was probably the major factor involved in the expression of the distinct features of PTC during secondary incubation on CUA.

The PTC characteristics of urease positive, 0.6-1.5 mm diameter, clear, colorless colonies possessing 1 or more differentiating usually dark blue or blue-green ring(s) about a usually lighter blue, blue-green or blue and white-marbled centre usually with a slight, clear colorless flat border or those convex 0.5-1.0 mm diameter less "mature" PTC colonies which appeared clear, colorless with a diffuse blue or pale-blue centre were believed to be partly the result of the alkaline reaction of these colonies with the bromothymol blue indicator in

Table 6

Results of comparison of recoveries of observed presumptive target (PTC) and presumptive background (PBC) colonies on membrane filters (MF) used to analyse various water samples after primary incubation of MF on Rimler-Shotts broth (RSB) or Rimler-Shotts agar (RSA) and secondary incubation on Christensen's urea agar (CUA)

Sample Source	Number of Samples	Number of MF analysed	Range of Volumes analysed (ml)	*Cumulative observed counts of PTC and PBC on MF on CUA after incubation on RSB or RSA			
				RSB		RSA	
				PTC	PBC	PTC	PBC
wells	8	8	5.0-50	1	702	0	383
lakes and streams	67	89	1.0-50	5	5,973	0	4,761
sewage	5	6	0.01-50	1	482	0	469
Totals	80	103	0.01-50	7	7,157	0	5,613

* Range of observed counts of PTC on individual MF was 0-3 colonies while range of observed PBC was 2 - 273 for RSB and 4 - 256 for RSA for individual MF. Median values for PBC on individual MF was 61 for RSB and 44 for RSA.

the RSB carried over with membrane filters when they were transferred to CUA for secondary incubation. PTC which developed as observable colonies only during secondary incubation may have been able to develop red urease reaction in CUA below the colony and a well defined slightly opaque centre but remained essentially colorless because they could not possibly have taken up the bromothymol blue indicator during primary incubation on RSB. On CUA much of this indicator had probably diffused away

from the filters and into the agar medium. For these reasons the use of RSB, rather than RSA, was recognized as an essential procedure in primary incubation for the subsequent development of the most distinctive types of PTC. Also, since even the non-blue colored PTC were not observed on filters treated by primary incubation on RSA it was assumed that other ingredients in the Rimler-Shotts media, which were required for the growth of Y.e. on CUA during secondary incubation, were not available for PTC development during secondary incubation of these filters but were available to the colonies on filters previously incubated on RSB. Further studies should be carried out to determine those ingredients used in the formulation of RSB which are essential for the development of Y.e. in the RSB-CUA system. Since the RSB-CUA system is the only procedure currently available for the quantitative determination of Y.e. aquatic samples efforts to determine the details of its working characteristics, with the view of improving the system, are highly recommended.

v) Recovery of Yersinia enterocolitica human isolate (serotype 0:3) from water suspension by membrane filtration

The calculated colony counts obtained from the analyses of dilutions of a water suspension of a human isolate (serotype 0:3) of Y.e. by the RSB-CUA system and by spiral plating are shown in Table 7. Also shown in Table 7 are the calculated counts for colonies recovered after primary and secondary incubation of membrane filters.

Owing to differences in the volumes analysed and the working surface area of membrane filters and agar plates the results obtained for the analyses of any of the dilutions of the original Y.e. suspension by the two methods are not directly comparable. For instance only the TSA plates used to analyse the 10^{-3} dilution yielded observed colony counts on all 5 replicate plates which were within the usually accepted plate counting range of 30 to 300 colonies. In this instance the actual observed counts on the 5 plates ranged from 116 to 148 colonies after 48 h incubation. Observed counts on the 5 TSA plates used to analyse the 10^{-4} dilution ranged from 9 to 21 colonies after the same incubation period. The observed counts of colonies on membrane filters used to analyse 50 ml of these dilutions, after secondary incubation, were too numerous to count (TNTC) for the 10^{-3} dilution and 214 colonies for the 10^{-4} dilution. The membrane filter used to analyse 50 ml of the 10^{-5} dilution bore a total of 21 colonies after secondary incubation. For purposes of comparing the recoveries of the human isolate of Y.e. from water suspension by the two methods, therefore, the calculated colony counts (expressed as a number per 100 ml of the original cell suspension) obtained by membrane filtration of either the 10^{-4} or 10^{-5} dilution and by spiral plating of the 10^{-3} dilution, probably afford the most representative data. These results suggest that the RSA-CUA system recovered only about 16% of the colonies recovered the spiral plating technique which employed an apparently less selective recovery medium, TSA.

Table 7

Comparison of calculated colony counts of a human *Yersinia enterocolitica* strain (serotype 0:3) recovered from water suspension by membrane filtration and spiral plating techniques

Dilution of original suspension analysed	Calculated colony counts expressed as number per 100 ml of original cell suspension		
	Membrane filtration with RSB-CUA system		Spiral Plating on TSA after 48 h at 35°C*
	After primary incubation	After secondary incubation	
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	5.8×10^5	TNTC	261×10^5
10^{-4}	7.4×10^5	42.8×10^5	300×10^5 **
10^{-5}	6.0×10^5	42.0×10^5	60×10^5 **
10^{-6}	10×10^5	20×10^5	no growth

TNTC - too numerous to count
* values calculated using mean of 5 replicates
** observed colony numbers on plates were not within acceptable counting range of 30-300 colonies

It is important to note, however, that in every case the colony counts obtained after primary incubation of filters on RSB increased greatly, by a factor of up to 7 times in some instances (Table 7), after the filters had been transferred to CUA and incubated for 48 h. This may suggest that RSB may contain substances which are inhibiting the growth of the strain of Y.e. examined, and perhaps of other strains as well, and that the inhibiting effect is reduced, likely by diffusion of the inhibitory substance(s) into the CUA, during secondary incubation.

Of major importance to the present assessment of the RSB-CUA system was the observation that the human isolate did not form colonies which were similar to those previously described as PTC on either RSB or later on CUA. After primary incubation, for instance, the recovered colonies were usually very small (pin-point to 0.3 mm diameter) as expected, but were mostly yellow in coloration or colorless. The yellow coloration indicates that the human strain (serotype O:3) used in this experiment rapidly utilized the maltose contained in RSB causing an acidification reaction and shift in color of the pH indicator from blue-green to yellow (Shotts & Rimler 1973). After secondary incubation most of these colonies had become pale-green or green in colour while some other usually smaller colonies appeared yellow. None of the colonies on any membrane filter appeared to produce an observable urease-positive reaction either during or after secondary incubation. Their green coloration, therefore, may be attributable mostly to the decarboxylation of ornithine by this bacterium which partially reversed the acid-yellow coloration due to maltose fermentation. Again, it appeared that ingredients contained in RSB which were carried over to CUA during transfer of the membrane filter for secondary incubation influenced the colony characteristics of this strain of Y.e.

Maltose fermentation is a characteristic of many but not all strains of Y.e. (Nilehn 1969) and the rate of maltose utilization

in many strains is markedly reduced at 37°C compared to that of cultures grown at 22°C. This human strain, therefore, probably was able to utilize maltose very readily at 35C and thus formed yellow rather than colorless colonies when incubated on RSB on membrane filters and this in turn may have prevented the expression of strong urease-positive reactions from occurring during secondary incubation of the filters on CUA. Since most strains of Y.e. are urease-positive, particularly when incubated at 35°C, the human strain examined may either have been an exception (Nilehn 1967) or the urease reaction was neutralized by this strain's ability to ferment maltose in RSB or glucose contained in CUA. Alternately some substances contained in RSB and carried over to CUA may have inhibited production of urease by this specific strain because the results of tests with this strain on slants of CUA and in the API-20E test system showed this strain to be strongly urease positive. If the RSB-CUA system is to be refined so that it reliably recovers all strains of Y.e. these phenomena should be studied more extensively.

The present study indicates that the RSB-CUA system probably would not recover a significant proportion of cells of human serotype O:3 Y.e. from water and that those recovered by this system might not be recognizable as PTC. Application of the RSB-CUA system to epidemiological investigation of outbreaks of yersiniosis due to strong maltose-fermentative strains or urease-negative strains therefore might not prove a suitable

epidemiological tool for water analyses. Results of extensive application of the system to surface water and wastewater analyses (Lautenschlager 1980), however, indicated that this system does recover a diverse population of different serotypes and biotypes of Y.e., some of which are serologically identical to strains of Y.e. which have been involved in human or animal infections (see Appendix B). Because of its potential to both recover and quantify Y.e. and related strairs in aquatic samples the RSB-CUA membrane filtration procedure should be carefully examined in an effort to improve its potential effectiveness in recovering strains of Y.e. which are most commonly involved in illnesses (e.g. Toma et al 1979).

vi) Determination of the specificity and selectivity of the RSB-CUA system for Yersinia enterocolitica

The results of calculations of gross presumptive selectivity indices for samples analysed in each month of the 1-year examination of surface and well water samples are presented in Table 8. Data obtained from the analyses of 545 water samples using 834 membrane filter determinations were used to calculate a gross presumptive selectivity index for the RSB-CUA system of 0.0151. This index suggests that the selectivity of the system applied to samples taken over the course of a 1-year period was very low. For instance this calculated index suggests that only 15 colonies of every 1,000 total colonies recovered by the system

would possess the colony characteristics of PTC. As can be seen in Table 8, the data are biased greatly by results obtained from many analyses conducted during the summer months when Y.e. was rarely detected in surface waters (Lautenschlager 1980). Presumptive monthly selectivity indices ranged from 0.1844 in February to 0.0000 for samples analysed in July and August. For this reason the selectivity indices were calculated using the same formula but excluding the data from analyses in which PTC were not recovered. The results of these calculations, as well as other relevant data, are recorded in Table 9.

By excluding the PTC-negative sample data the gross presumptive selectivity index was calculated to be 0.0410, nearly 3 times greater than was determined by the former procedure. Also, the monthly presumptive selectivity indices for several months in which PTC were recovered from samples are increased significantly by excluding PTC-negative sample results from the calculations.

Data used to determine the confirmed selectivity indices of 12 surface water samples analysed in March and April of 1979 are recorded in Table 10. The cumulative totals of confirmed PTC, confirmed PBC, false PTC and false PBC were used to calculate the confirmed selectivity index of the RSB-CUA system used for these analyses by the formula:

Table 8

Frequency of detection of presumptive target colonies (PTC), cumulative numbers of PTC, presumptive background colonies (PBC) and total colonies, and presumptive selectivity indices for analyses of all surface water samples analysed using the Rimler-Shotts Urea Agar (RSB-CUA) procedure during the period February 1979 to January 1980.

Month of Year	#Samples[1] Analysed	% #Samples Yielding PTC	#MF Determinations	Cumulative numbers of colonies observed on all MF's used for analysis			Presumptive Selective Index
				PTC	PBC	TOTAL*	
Feb. 79	7	100	9	71	314	385	0.1844
Mar. 79	14	100	16	61	350	411	0.1484
Apr. 79	22	100	37	210	1,248	1,458	0.1440
May 79	101	64.4	188	202	11,679	11,881	0.0170
June 79	228	30.2	378	167	22,998	23,165	0.0072
July 79	68	1.5	78	1	6,826	6,827	0.0001
Aug. 79	53	0	62	0	5,142	5,142	0.0000
Sep. 79	4	0	4	0	324	324	0.0000
Oct. 79	12	41.7	16	16	2,362	2,378	0.0067
Nov. 79	10	50.0	9	15	1,247	1,262	0.0119
Dec. 79	16	81.3	25	46	1,390	1,436	0.0320
Jan 80	10	70.0	12	44	537	581	0.0757
TOTALS	545	▲38.1	834	833	54,417	55,250	0.0151

* PTC plus PBC
▲ 208 PTC - positive samples
1 An additional 55 samples did not yield data with absolute values of either PTC or PBC so have not been considered in this table.

Table 9

Water temperature, frequency of detection of presumptive target colonies (PTC), cumulative numbers of PTC and presumptive background colonies (PBC) observed on membrane filters (MF) and presumptive selectivity indices for analyses of surface water samples containing PTC using the Rimler-Shotts-Urea agar procedure during the period February 1979 to January 1980

Month of Year	Median Water Temperature (°C)	Frequency of Detection of PTC in all Samples (%)	Number of Samples in Which PTC Detected	Number of MFs on Which PTC Observed	Cumulative numbers of colonies observed on all MF's bearing PTC			Presumptive Selectivity Index
					PTC	PBC	TOTAL	
Feb. 79	1.0	100	7	9	71	314	385	0.1844
Mar. 79	2.0	100	14	16	61	350	411	0.1482
Apr. 79	7.5	100	22	37	210	1,248	1,458	0.1440
May 79	13.9	64.4	65	83	202	7,404	7,606	0.0266
June 79	18.8	30.2	69	91	167	7,236	7,403	0.0256
July 79	23.1	1.5	1	1	1	29	30	0.0333
Aug. 79	19.9	0	0	0	-	-	-	-
Sep. 79	12.8	0	0	0	-	-	-	-
Oct. 79	10.0	41.7	5	5	16	1,175	1,191	0.0134
Nov. 79	5.9	50.0	5	7	15	710	725	0.0207
Dec. 79	2.4	81.3	13	13	46	602	648	0.0710
Jan 80	2.8	70.0	7	9	44	409	453	0.0971
TOTALS		20.2	208	271	833	11,477	20,310	0.0410

$$\frac{\text{Total Confirmed Target}}{\text{Total Confirmed Target + Confirmed Background + Total False PTC}} = \text{Confirmed Selectivity Index}$$

For these 12 analyses the confirmed selectivity index was calculated to be: (using data in Table 10)

$$\frac{(32)}{(32 + 141 + 1)} = \frac{32}{174} = 0.1839$$

Only 1 false PTC was identified and this culture found to be a species of Pseudomonas. Of the 9 false PBC identified as Y.e. 8 were present on membrane filters after secondary incubation in the RSB-CUA system as clear, colorless colonies 0.2 - 0.4 mm in diameter. These, therefore, were probably Y.e. colonies which grew slowly or developed late in the secondary incubation period and were considered to be immature colonies. The other false PBC was observed to be a small very pale brown colony after secondary incubation. While this latter calculation of the confirmed selectivity index of the RSB-CUA system suggests that about 18% of all colonies recovered with this system in the analyses of surface waters in March and April will be Y.e., and agrees well with presumptive selectivity indices for analyses conducted in

Table 10

Results of confirmatory analysis of all presumptive target (PTC) and all presumptive background (PBC) colonies observed on membrane filters used to analyse various volumes of 12 surface water samples using the RSB-CUA procedure

Sampling Date (1979)	Volume Analysed (ml)	Number of PTC				Number of PBC			
		Observed at 48 h	Lost Before Identi-fication	Confirmed as Target	Confirmed as False PTC	Observed at 48 h	Lost Before Identi-fication	Confirmed as Background	Confirmed as False Background
March 12	50	2	0	2	0	22	1	19	2
March 19	1.0	1	0	1	0	15	7	8	0
March 19	1.0	1	0	1	0	3	2	1	0
March 19	1.0	1	0	1	0	17	8	9	0
March 19	50	13	0	13	0	18	7	11	0
March 26	1.0	2	0	2	0	10	4	5	1
March 26	10	0	0	0	0	12	6	4	2
March 26	10	1	0	1	0	16	2	11	3
April 2	1.0	2	1	1	0	5	5	0	0
April 2	1.0	6	1	5	0	14	7	7	0
April 2	50	2	1	1	0	9	4	4	1
April 9	10	2	1	0	1	9	2	7	0
TOTALS		33	4	28	1*	150	55	86*	9

Confirmed background colonies included 26 Enterobacter spp; 21 Pseudomonas spp; 5 Citrobacter spp; 4 Serratia sp; 2 Aeromonas hydrophila; 1 each of Achromobacterium sp; Acinetobacter sp; Alcaligenes sp; Pasteurella multicida and 24 colonies which could not be identified but which werenon-target in their biochemical characteristics in API-20E.

* The single false-PTC was identified as Pseudomonas sp. having an API-20E profile number of 0002004-51.

March and April (Table 9), it shows the system lacks selectivity. In summer months when surface water temperatures rise above 10-15°C and PBC counts increase relatively greatly the confirmed selectivity index of the system would probably be very low.

Despite the low selectivity of the RSB-CUA system when calculated on a monthly or seasonal basis numerous samples were analysed, particularly during the months of ice-cover on lakes, from which PTC were observed to comprise up to 50% of the total colony count observed on individual membrane filters. This high proportion of PTC is more due to the absence of PBC in water at that time of year than to any selective characteristic of the RSB-CUA system. For the same period, in 1979 data was recorded concerning 3 samples obtained from sewage lagoon systems. These data are shown in Table 11. The confirmed selectivity index calculated on the basis of the data in Table 11 indicates a confirmed selectivity index of 0.2083, similar to that obtained for lake water analyses for the same time of year using the RSB-CUA system. In actual practice, however, the analysis of sewage wastewater samples was usually not a simple matter as most of the other samples were heavily contaminated with PBC, making enumeration of PTC difficult, but still permitting estimates of the PTC population to be made. In these cases the estimates of PTC were considered to be underestimates of the actual PTC population in sewage.

Table 11

Results of confirmatory analysis of all presumptive target (PTC) and all presumptive background (PBC) colonies observed on membrane filters used to analyse various volumes of 3 drainage water or wastewater samples from sewage lagoon systems using the RSB-CUA procedure.

Sample Source	Volume Analysed (ml)	Number of PTC				Number of PBC			
		Observed at 48 h	Lost before Identi-fication	Confirmed as PTC	Confirmed as False PTC	Observed at 48 h	Lost Before Identi-fication	Confirmed as Background Colonies*	Confirmed as False PBC
Drainage Water	1.0	5	1	4	0	15	2	9	4
Drainage Water	1.0	3	0	3	0	15	5	10	0
Lagoon Waste-Water	0.1	2	0	2	0	12	10	2	0
TOTALS		10	1	9	0	42	17	21*	4

* Confirmed background colonies identified as 8 Enterobacter spp; 5 Pseudomonas spp; 3 Citrobacter sp; 2 Serratia spp; 1 Flavobacterium sp. and 2 could not be identified but were non-target in their biochemical characteristics in API-20E.

An estimate of the specificity of the RSB-CUA system was calculated using the results of identification tests conducted on 633 PTC recovered from water and wastewater sample analyses in 1979 and 1980. Eighty-six per cent of these PTC were identified and confirmed as isolates of Y.e. Of the 633 PTC subjected to identification procedures a total of 86 (14%) were identified as false PTC. Of these false PTC, 34 could not be identified using the API-20E biochemical test system. The other false PTC which were identified were 12 strains of Enterobacter spp, 9 strains of Klebsiella spp, 8 strains of Aeromonas hydrophila, 6 strains each of Hafnia alveii and Pseudomonas spp, 4 strains each of Providencia sp and Serratia spp, 2 strains of Citrobacter sp, and 1 strain tentatively identified as Acinetobacter sp.

As indicated in Tables 10 and 11 Y.e. were frequently detected among the PBC and these were nearly always colonies which were considered to be immature or late developing strains of Y.e. in that they were usually small, clear and colorless. Previous observations made during the course of this study indicated that many of these immature colonies would develop the characteristics of distinct PTC if the secondary incubation period for membrane filters incubated on CUA was extended to 72 or to 96 h. The increase in recovery of confirmed Y.e. by adopting this extended incubation procedure was not considered to be sufficient to justify the delays in obtaining counts of PTC that would be associated with extended periods of secondary incubation.

Most (73) of the 86 false PTC were recovered on membrane filters used to analyse water samples during the months of May, June and July. That these non-Y.e. false PTC were more frequently encountered during this period may reflect both the increased density of the species comprising the false PTC population in warmer water and the effects of crowding of colonies on membrane filters when analyses were conducted on samples with higher background to target colony ratios.

Of the 547 isolates confirmed as Y.e. by the API-20E biochemical test system a total of 182 (Appendix B) were submitted to the Yersinia Reference Laboratory for serological and biochemical characterization. All 182 isolates were re-confirmed as Y.e. or closely related species. Thirty-one per cent (57) of these showed biochemical characteristics which were "typical" for various biotypes of Y.e. while the remainder differed from typical Y.e. mainly with regard to their reactions to rhamnose, citrate, sucrose or in their Voges-Proskauer reaction. Forty per cent (73) of the isolates submitted were serotypable with one or more of the 34 Y.e. antisera employed for typing of Y.e. at the Reference Laboratory (Appendix B). While these results indicate that the RSB-CUA system recovers a very diverse population of Y.e. and Y.e.-like bacteria from water we did not observe any morphological features of PTC recovered by the system which would be of assistance in visually differentiating between the various serotypes recovered. In a future report these results will be

re-interpreted using the more recent taxonomical classification schemes (eg. Ursing et al 1980; Shayegani et al 1981) which permit differentiation of Yersinia enterocolitica sensu stricto from the Y.e.-like bacteria (eg. Y. intermedia, Y. frederiksenii, Y. kristensenii etc.).

These results indicate that the RSB-CUA system is subject to a relatively severe lack of selectivity when applied to water analyses during periods of warm water temperature. However, at all times the system, when used strictly within the confines of the PTC description, provides for a relatively highly specific system for the determination of Y.e. in surface, well and wastewaters. With disciplined systematic research this membrane filtration procedure could likely be markedly improved to recover specific strains of Y.e. from many different and highly contaminated environmental aquatic samples. It will require considerable research, however, to determine the effects of the media and incubation regimes used in the RSB-CUA system upon the retention of factors responsible for the expression of virulence in strains of Y.e. recovered by these procedures.

Summary

Experiments were conducted to assess the use of several procedures for the routine analyses of water samples for the bacterium, Yersinia enterocolitica. Isolates of Y.e were obtained from lake water and from the feces of beaver using an urea deoxycholoate broth (UDB) enrichment and a cold-enrichment procedure with MacConkey Tween 80 agar as selective isolation media. Aerobic incubation of UDB at 20°C was considered to provide the best non-quantitative enrichment procedure for recovering Y.e. from water on subsequently used isolation media. Cold-enrichment of fecal samples for 6 weeks prior to enrichment of fecal-cultures in UDB were required to recover Y.e. from beaver. The first known strain of Y. ruckeri obtained from an enteric mammalian source was isolated from muskrat feces using UDB enrichment. The Y. ruckeri isolate was subsequently shown to be virulent to salmonid fishes by other workers.

A two-step membrane filtration procedure for the detection and enumeration of Y.e. in surface waters and in wastewaters was developed. Attempts to improve the selectivity of this RSB-CUA system of membrane filtration analyses by altering incubation regimes, and concentrations of antibiotic or inhibitors in the RSB primary isolation medium were not successful. Presumptive selectivity of the system was low and varied seasonally with the populations of target and background bacteria present in water at various times of the year, and was highest for all analyses conducted during winter months. Confirmed selectivity of the system was similarly low indicating that in most analyses less than 20% of the total colonies

recovered from water would be confirmed as Y.e. target colonies. Based on the results of identification procedures involving 633 presumptive target colonies (PTC) obtained from analyses conducted over a 1-year period approximately 86% of observed presumptive target colonies were confirmed as Y.e. Most false PTC were recovered from water analyses conducted during periods of warm (>10-15°C) water temperature. Y.e. was also frequently detected as small, clear colorless colonies among presumptive background colonies observed on membrane filters. Recommendations for further studies to improve the selectivity of the media and procedures used in the RSB-CUA system are presented.

Recommendations

1. Major efforts should be made to modify the RSB-CUA system to permit maximum recovery of strains of Y.e. as distinct PTC at incubation temperatures of 30°C or less. This might best be achieved by careful examination of the addition of selective inhibitory agents to the media employed in the system.

2. Studies should be carried out to determine the potential health significance of Y.e. strains isolated by the RSB-CUA system.

3. Concurrently with attempts to improve upon the selectivity and specificity of the RSB-CUA system and to determine the accuracy of the system in recovering Y.e. from water the system should be further applied to the analyses of surface, drinking and wastewaters in order to expand upon current knowledge of the distribution and ecology of Y.e. and Y.e.-like bacteria in aquatic environments.

4. Laboratories adopting the RSB-CUA system for determination of Y.e. in water or wastewater would benefit greatly by including in their research, at least initially, the mapping and identification procedures shown in the example in Appendix A. This would provide technicians with a sound knowledge of recognizing Y.e. and various PBC species with a high degree of proficiency.

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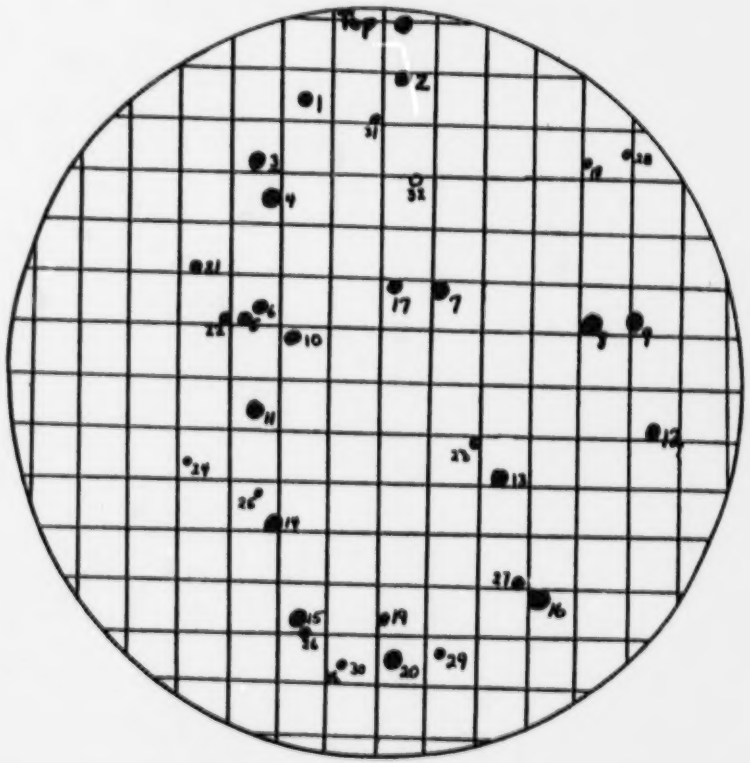
APPENDIX A

Example of mapping and recording procedures used in the development of the membrane filtration RSB-CUA system.

APPENDIX A

Figure 1 Example of mapping system used to record colony positions and development* on membrane filters during incubation on RSB and CUA.

DATE: March 19, 1979
SAMPLE: Grandview L. - outflow
SAMPLE VOLUME: 50 ml



- colonies 1-14 observed after primary incubation 20 March 79.
- colonies 15-21 first observed after 24 hrs on CUA 21 Mar 79.
- colonies 22-31 first observed after 48 hrs on CUA 22 Mar 79. Colony 32 not examined

Cultures 1229 - 1259 retained in frozen storage

Colonies 1, 3, 4, 7, 9, 11, 12, 14, 16 & 19 confirmed as *Y. enterocolitica*

X - contaminant debris

* Detailed colony descriptions and culture characteristics are recorded in Tables 1 and 2 of this Appendix.

APPENDIX A

Table 1

Example of data sheets used
to record visual observations
of colonies during incubations
of membrane filters using the
RSB-CUA system.*

* copied from handwritten originals for the
Grandview L. analyses depicted in Figures 1 and 2

SAMPLE SOURCE: Grandview Lake STATION: Outflow SAMPLING DATE: March 19, 1979
SAMPLE VOLUME: 50 ml

Observation Date: March 20, 1979				Observation Date: March 21, 1979			Observation Date: March 22, 1979		
Colony No. on Grid Map	Lab. Culture Number	Colony Characteristics on RSB at 22±2hrs		Colony Characteristics on CUA at 24 hrs.			Colony Characteristics on CUA at 48 hrs.		
		Colour, Shading, Border, Contour, etc.	Colony Size (mm)	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction
1	1229	colorless	< .3	clear, very pale yellow or colorless	0.7	-	clear, blue-ringed pale blue centre with clear colorless border	0.9	obscured
2	1230	colorless with very slight pale yellow tinge	.5	yellow	1 mm	-	clear, yellow-green	1.1 mm	"
3	1231	colorless	.4	clear, pale diffuse blue centre with colorless border	1 mm	+	clear, blue-ringed pale blue centre, with colorless border	1.2	"
4	1232	colorless	.5	clear, pale blue centre with colorless border	1.1	+	clear, blue-ringed pale blue centre, with colorless border	1.5	"
5	1233	colorless	< .3	pale (clear) yellow	0.9	-	clear, blue-ringed pale blue centre, with colorless border	1.0	"
6	1234	colorless	< .3	clear, pale yellow	0.71	-	clear, pale green	1.0	"
7	1235	colorless	.5	clear, pale blue centre with colorless border	1.0	+	clear, blue-ringed pale blue centre, with colorless border	1.5	"
8	1236	colorless with green tinge	≤ .75	clear, pale yellow	1.3	-	clear, yellow green	1.4	"
9	1237	colorless	.3	colorless, clear distorted by black line on filter	< 1.0 distorted by line	+	clear, blue-ringed pale blue centre, with colorless border	1.1	"

SAMPLE SOURCE: Grandview Lake
SAMPLE VOLUME: 50 ml

STATION: Outlfow

SAMPLING DATE: March 19, 1979

Observation Date: March 20, 1979				Observation Date: March 21, 1979			Observation Date: March 22, 1979		
Colony No. on Grid Map	Lab. Culture Number	Colony Characteristics on RSB at 22±2hrs		Colony Characteristics on CUA at 24 hrs.			Colony Characteristics on CUA at 48 hrs.		
		Colour, Shading, Border, Contour, etc.	Colony Size (mm)	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction
10	1238	colorless with pale yellow-yellow green tinge	≤ .5	clear, pale yellow	1.0	-	clear, pale green	1.2	obscured
11	1239	colorless	.6	clear, pale blue centre with colorless border	1.0	+	clear, blue-ringed pale blue centre with colorless border	1.4	"
12	1240	colorless	.4	clear, very pale blue tinge to centre, colorless border	0.9	+	clear, pale blue-ringed very pale blue centre with colorless border	1.1	"
13	1241	colorless	.4	clear, very pale blue centre, with colorless border	0.8	+	clear, blue-ringed pale blue centre with colorless border	1.0	"
14	1242	colorless	.4	clear, very pale blue centre with colorless border	0.7	+	clear, blue-ringed pale blue centre with colorless border	1.0	"
15	1243	N.O.		clear, yellow	0.9	-	clear, yellow green	1.0	"
16	1244	N.O.		clear, very pale blue centre with colorless border	1.1 mm	-	clear, blue-ringed pale blue centre with colorless border	1.2	"
17	1245	N.O.		clear, colorless	0.5	-	clear, pale yellow	0.8	"

N.O. = not observed

SAMPLE SOURCE: Grandview Lake STATION: Outflow SAMPLING DATE: March 19, 1981 3 of 4
SAMPLE VOLUME: 50 ml.

Observation Date: March 20, 1979				Observation Date: March 21, 1979			Observation Date: March 22, 1979		
Colony No. on Grid Map	Lab. Culture Number	Colony Characteristics on RSB at 22±2hrs		Colony Characteristics on CUA at 24 hrs.			Colony Characteristics on CUA at 48 hrs.		
		Colour, Shading, Border, Contour, etc.	Colony Size (mm)	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction
18	1246	N.O.		clear, colorless	0.4	-	clear, pale yellow	0.6	obscured
19	1247	N.O.		clear, colorless	0.4	-	clear, differentiated ring about colorless centre with pale blue dot at centre	0.8	"
20	1248	N.O.		clear, colorless	0.6	-	clear, differentiated ring about pale blue centre	1.0	"
21	1249	N.O.		clear, colorless	0.3	-	clear, pale yellow	0.8	"
22	1250	N.O.					clear, pale yellow-green	0.3	"
23	1251	N.O.					clear, colorless	0.3	"
24	1252	N.O.					clear, colorless	0.2	"
25	1253			N.O.			clear, colorless	0.2	"
26	1254			N.O.			clear, pale yellow	0.2	"
27	1255			N.O.			clear, very pale yellow	0.3	"
28	1256			N.O.			clear, pale yellow	0.4	"

N.O. = not observed

SAMPLE SOURCE: Grandview Lake
SAMPLE VOLUME: 50 ml

STATION: Outflow

SAMPLING DATE: March 19, 1979

Observation Date: March 20, 1979				Observation Date: March 21, 1979			Observation Date: March 22, 1979		
Colony No. on Grid Map	Lab. Culture Number	Colony Characteristics on R-S at 22±2hrs		Colony Characteristics on CUA at 24 hrs.			Colony Characteristics on CUA at 48 hrs.		
		Colour, Shading, Border, Contour, etc.	Colony Size (mm)	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction	Color, Shading, Border, Contour, etc.	Colony Size (mm)	Urease Reaction
29	1257	———	———	N.O.	———	———	clear, pale yellow	0.3	obscured
30	1258	———	———	N.O.	———	———	clear, pale yellow	0.3	"
31	1259	———	———	N.O.	———	———	clear, colorless	0.3	"

N.O. = not observed

* only colonies 1-14 observed after primary incubation on RSB
** colonies 15-21 first observed after 24hrs on CUA during secondary incubation
*** colonies 22-31 first observed after 48hrs on CUA during secondary incubation

APPENDIX A

Table 2

Example of data sheets used
to record biochemical, serological and
morphological characteristics of
colonies recovered using the
RSB-CUA system.*

* copied from handwritten originals for the
Grandview L. analyses depicted in Figures 1 and 2

SAMPLE SOURCE: Grandview Lake STATION: Outflow SAMPLING DATE: March 19, 1979
SAMPLE VOLUME: 50 ml

Lab. Culture Number	Colony Descriptions and Main Features on MT80 Agar						Screen Test Reactions								Confirmation Results			
	25C			35C			Oxidase Reaction	TSI Slant				M.S.M.		CUA Slant	API 20E Number	API 20E Identification	Sero-Type	Bio-Type
	LAC	LIP	Features	LAC	LIP	Features		Slant	Butt	Gas	H_2S	25C	35C					
1229	+	+	purple, raised flat hollow centres, wavy border, smooth, glossy	-	-	very pale pink, raised flat, texture wrinkled (grooves)	-	A	A	-	-	-	+ slight H_2S	day 1-slight & butt day 4-entire	1155523	Y. enterocolitica (excell I.D.)	17	1
1230			spreader, whitish pink	-	-	peak, convex, entire, smoooth dull gloss	+	K	K	-	-	+	+	day 1-entire	0201004-41	Alcaligenes species (acceptable I.D.)	-	-
1231	+	+	purple, raised flat, hollow centres, wavy border, smooth, glossy	-	-	white, convex (some wrinkled) entire smooth, dull, gloss	-	A	A	-	-	+	+	day 1-slight & butt day 4-entire	1154733	Y. enterocolitica	non-typable	1
1232	+	+	purple, raised flat, hollow centres, wavy border, smooth, glossy	-	-	peach, raised, flat, wrinkled texture	-	A	A	-	-	+	+	day 1-slight & butt day 4-entire	1154523	(excellent I.D) Y. enterocolitica	non-typable	1
1233	+	+	purple, raised flat, hollow centres, wavy, smooth, glossy	-	-	peach, raised flat, wrinkled	-	A	A	-	-	+	+	day 1-slight & butt day 4-entire	1155733	1) Yersinia ent. 2) Kl oxytoca 2) S. liquefaciens	non-typable	1
1234	+	+	whitish pink, drop-like	+	-	pink, convex, entire, smooth dull, gloss	+	K	K	-	-	+	slight near top of tube	day 1-entire	2203004-41	(very good I.D.) Ps. species	-	-
1235	+	+	purple, raised flat, hollow centres, wavy, smooth, glossy.	-	-	peach, raised flat-wrinked, but some are smooth	-	A	A	-	-	+	+	day 1-slight & butt day 4-entire	1155723	(excellent I.D.) Y. enterocolitica	non-typable	1
1236	+	-	red, hilly, irregular border, flamentous, dull	+	-	purple, hilly	-	not inoculated				-	+	day 1-entire	1205101	1) E. Agglomerans 2) S. rubidae (questionable I.D.)	-	-
1237	+	+	purple, raised-flat, hollow centres, wavy, smooth, glossy	-	-	white, raised flat, wrinkled	-	A	A	-	-	+	+	day 1-slight & butt day 4-entire	1155723	(excellent I.D.) Y. enterocolitica	non-typable	1
1238	+	-	light purple, raised flat, hollow centre irregular, dull, grainy	+	-	purple convex with a ridge around centre, flamentous ridge	-	K	A	-	-	very slight	+	day 1-entire	0205101 repeated-same result	Doubtful Profile	-	-
1239	+	-	dull whitish pink, flat-convex scalloped smooth, glossy	-	-	peach, convex entire, smooth, dull gloss	-	A	A	slight	-	+	very slight	day 1-slight & butt day 4-entire	1155723	Y. enterocolitica (excellent I.D.)	non-typable	1

A = acid, K = alkaline, N.C. = No change, Lac = lactose fermentation; Lip = Lipolysis; N.T. = non-typable

SAMPLE SOURCE: Grandview Lake STATION: Outflow SAMPLING DATE: March 19, 1979
SAMPLE VOLUME: 50 ml

Lab. Culture Number	Colony Descriptions and Main Features on MT80 Agar						Screen Test Reactions								Confirmation Results			
	25C			35C			Oxidase Reaction	TSI Slant				M.S.M.		CUA Slant	API 20E Number	API 20E Identification	Sero-Type	Bio-Type
	LAC	LIP	Features	LAC	LIP	Features		Slant	Butt	Gas	H_2S	25C	35C					
1240	+	-	whitish pink, raised flat, (contaminated)	sl. +	-	light pink, raised flat, small hollow centres, entire smooth, dull gloss	-	A	slight A	-	-	+	-	day 1-slight & butt day 4-entire	1155523	Y. enterocolitica (excellent I.D.)	ND	ND
1241	+	+	light purple, raised flat, hollow-centres, wavy, smooth gloss	-	-	very light pink, convex, wrinkled	-	A	A	slight	-	-	+	day 1-slight & at butt day 4-entire	1155773	1) Y. enterocolitica 2) K. oxytoca 3) S. liquefaciens	ND	ND
1242	+	+	pink, raised flat	-	-	peach, convex entire smooth, dull gloss	-	A	A	slight	-	+	+	day 1 - + butt day 4 - entire	1155523	Y. enterocolitica (excellent I.D.)	ND	ND
1243			spreader, whitish	+	-	dark pink, convex entire smooth, dull gloss	+	K	K	-	-	+	slight at top of tube	day 1 - entire	2207004	(very good I.D.) Ps fluorescens gr.	-	-
1244	+	+	Purple, raised flat, hollow centres, wavy, smooth, glossy	-	-	slight pink, convex entire, smooth dull gloss	-	A	A	-	-	+	+	day 1 - + butt day 4 - entire	1155523	Y. enterocolitica (excellent I.D.)	ND	ND
1245	-	-	white, flat, entire smooth, dull	-	-	white with a tinge of pink, convex entire, smooth	+	K	K	-	-	+	+	day 1 - entire	2203004-41	Ps species (very good I.D.)	-	-
1246	-	-	white, flat, contaminated with yellow colonies	-	-	slight pink, convex, centre, smooth	-	K	K	-	-	+	slight +	day 1-entire	0210000-41	1) CDC group 4C2 2) Ps. maltophila 3) B.bronchioseptica	-	-
1247	+	+	purple, raised flat hollow-centre wavy, smooth, glossy	-	-	slight pink, convex wrikled	-	A	A	-	-	+ slight H_2S at top of tube	slight +	day 1 - + butt day 4 - entire	1155723	Y. enterocolitica (excellent I.D.)	ND	ND
1248	+	+	pink, raised flat, hollow-centres, wavy, smooth, glossy	-	-	slight pink, convex wrinkled	+	A	A	-	-	+	+	day 1 - + butt day 4 - entire	1155733	1) Y. enterocolitica 2) K. oxytoca 3) S. liquefaciens	ND	ND
1249	-	-	off white, flat	-	-	pink, flat		K	slight A	+	-	-	not inoc-ulated	day 1 - + butt day 4 - entire	0213004-01	1) B.bronchioseptica 2) Alcaligenes sp. 3) Pseudomonas sp.	-	-
1250	-	-	white, hilly	-	-	slight pink, convex entire, smooth, glossy	-	ND	ND	ND	ND	ND	+		4202000-41	P. maltopila (excellent I.D.)	-	-

A = acid, K = alkaline, N.C. = No change, Lac = lactose fermentation; Lip = Lipolysis, N.T. = non-typable N.D. = not determined

SAMPLE SOURCE: Grandview Lake STATION: Outflow SAMPLING DATE: March 19, 1979
SAMPLE VOLUME: 50 ml

Lab. Culture Number	Colony Descriptions and Main Features on MT80 Agar						Screen Test Reactions								Confirmation Results			
	25C			35C			Oxidase Reaction	TSI Slant				M.S.M.		CUA Slant	API 20E Number	API 20E Identification	Sero-Type	Bio-Type
	LAC	LIP	Features	LAC	LIP	Features		Slant	Butt	Gas	H_2S	25C	35C					
1251			no growth															
1252	-	-	white convex, irregular, bumpy			slight pink, convex, smooth	+	K	slight A	-	-	-	slight at top of tube	day 1-entire	2200004-51	1) Pseud opp. other 2) Ps. putida 3) P. flourescens		
1253			no growth			didn't grow												
1254	-	-	light pink, flat			didn't grow												
1255	-	-	light pink, flat	+	-	pink, convex, entire smooth, glossy	redo, no growth last attempt	A	A	+	-	slight + H_2S	+ H_2S	day 1 - entire		to repeat; unacceptable I.D. on second attempt		
1256	-	-	light pink, flat	+	-	pink, convex, entire smooth, glossy	redo, as well	slight A	A	-	-	+	+	day 1 - entire		ND		
1257	-	-	light pink, flat			didn't grow												
1258	-	-	light pink, flat			didn't grow												
1259	-	-	white, hilly			didn't grow												

A = acid, K = alkaline, N.C. = No change, Lac = lactose fermentation; Lip = Lipolysis; N.T. = non-typable N.D. = not determined

APPENDIX A

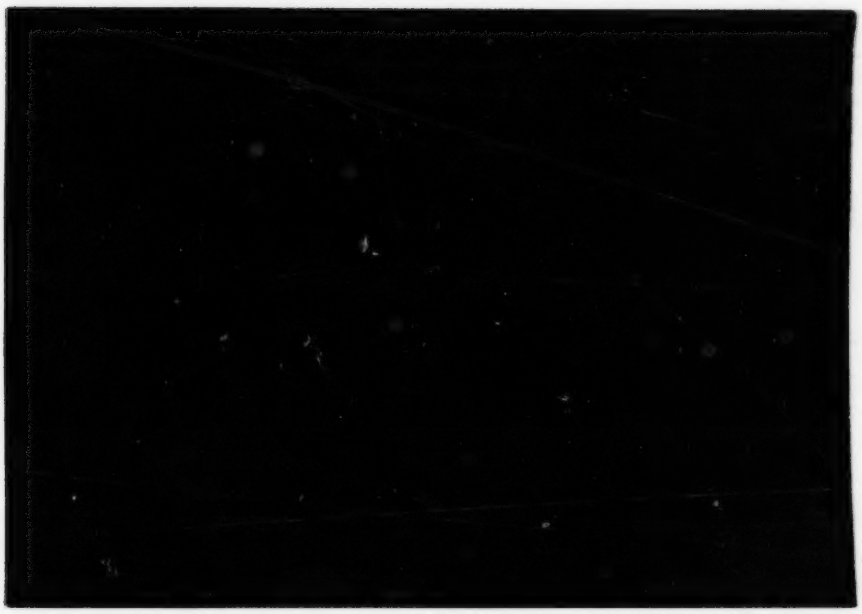


Figure 2 Photograph of a membrane filter used to analyse 50 ml of water from Grandview Lake on March 19, 1979 using the RSB-CUA system. The blue-centered colonies are strains of Y. enterocolitica. Details of the colonies' development and biochemical characteristics can be found using Figures 1 and 2 and the corresponding information recorded in Tables 1 and 2 of this appendix.

APPENDIX A

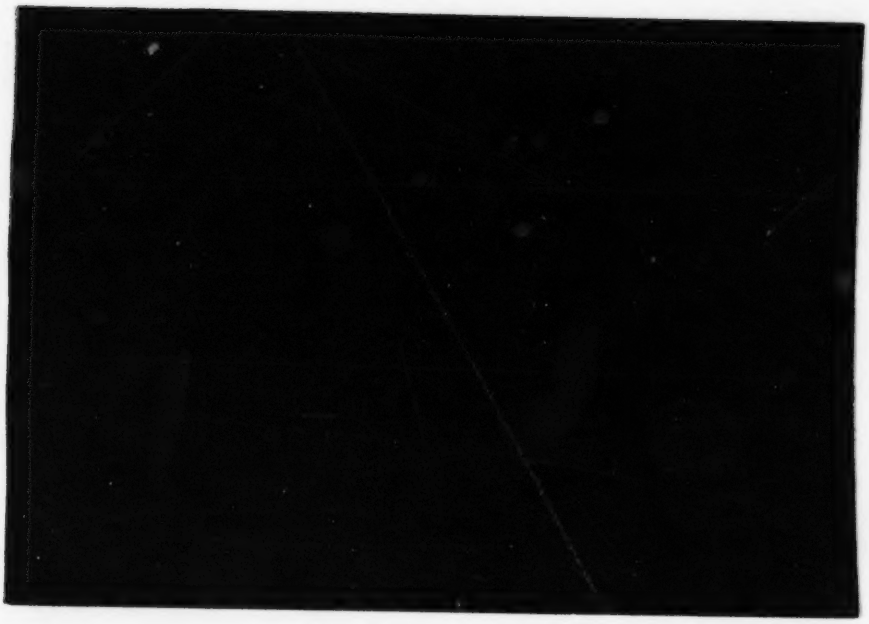


Figure 3 Photograph of a membrane filter (MF) used to analyse 25 ml of lake water on May 8, 1979. Secondary incubation of the MF in this case was on modified urea agar (mCUAM) for 48 h. The blue-centered colonies were confirmed as Y. enterocolitica.

APPENDIX A

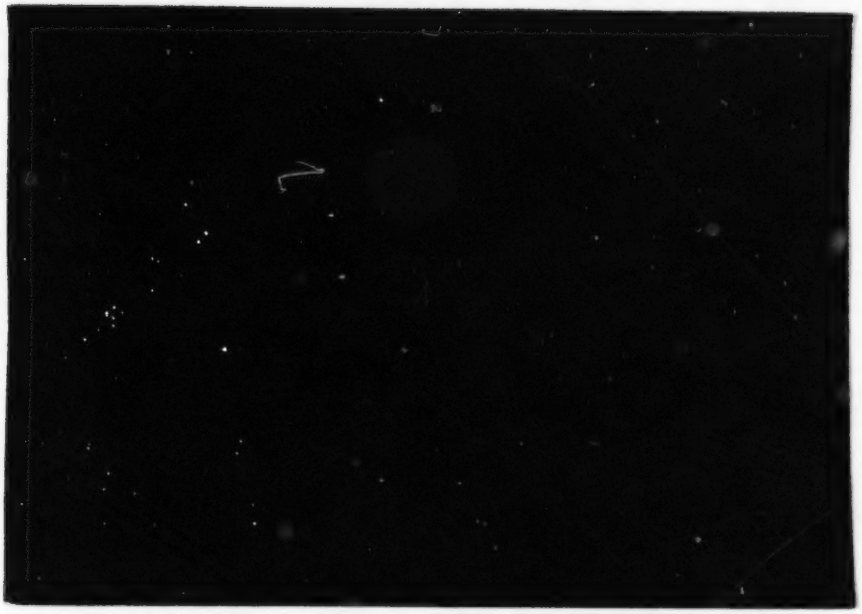


Figure 4 Photograph showing a membrane filter (MF) used to analyse 0.01 ml of sewage lagoon waste water in February of 1979. Eleven colonies of Y. enterocolitica, mostly possessing presumptive target colony features, were obtained from this MF. The large (5 mm diameter) colony in the lower left is Klebsiella pneumoniae.

Figure 5a

Figure 5b

Figure 5 Photographs of Y. enterocolitica (Y.e.) colonies grown on MacConkey Tween 80 (MT80) agar after incubation for 48 h at 25°C. Two Y.e. colonies are apparent in the central part of Figure 5a as small, lipolytic colonies with concave centres. These were recovered from a cold-enrichment suspension of beaver feces inoculated directly onto MT80. Figure 5b shows the distinct concave centres of colonies of a pure culture of a water isolate of Y.e. on MT80 at 25°C. Y.e. isolates did not display this feature when grown on MT80 at 35°C.

APPENDIX B

Serological and Biochemical Characteristics of

Yersinia enterocolitica Isolated from Water and

Sewage using the RSB-CUA Membrane Filtration Procedure

APPENDIX B

Serological and Biochemical Characteristics of Yersinia enterocolitica isolated from water and sewage using the RSB-CUA membrane filtration procedure.

"O" Serotype	Biotype	Number of Cultures of each Serotype	Number of serotypable cultures showing various atypical or typical biochemical reactions				
			R+	C+	R+C+	S-VP-	Typical
4, 32	1	2	1				1
4, 33	1	10	3	1	3		4
5	1	2					2
6, 30	1	1					1
6, 31	1	4					4
7, 8	1	3					3
11, 23	1	1				1	
11, 24	1	3				2	1
11, 14	2	1				1	
12	2	1					1
14	1	4	2	1	1		
14, 16	1	5	2		2		1
16	1	19	11				8
16, 29	1	1					1
17	1	9	3	2	2		2
18	1	4			1		3
21	1	2*			1		1
34	1	1			1		
N.T.	1, 2 & 3	89**	25	9	30	1	24
TOTALS		162	47	13	41	5	57

R+ = rhamnose +, C+ - citrate +, S-VP- = sucrose and Voges-Proskauer-,
* 5 isolates of serotype O:21 (R+) were recovered from feces of beaver after cold enrichment
** an additional 15 N.T. cultures not described above displayed a variety of reactions and combinations of the above reactions
N.T. = nontypable